Detection and control of T. brucei s.l. in the historic sleeping sickness foci of NW Uganda

Thesis submitted in accordance to the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Lucas J. Cunningham

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Dedication

I would like to dedicate this work to my family, especially my wife and the next project in my life, our baby girl.

To Natalie Louise Wood-Cunningham and Otylia Ana Cunningham

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Over the past three-plus years of carrying out this project I have had the pleasure of working with some of the best and brightest people in the field of tropical diseases. I would not have gotten to the point I am at now without their help, support and in many cases patience.

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Declaration of work done

This thesis combines work carried out by the author and a number of collaborators, this page serves to detail the division of labour amongst the participants.

Chapter 2- The development and design of the novel primers in this thesis were developed by the author under advice from Prof Martin Donnelly and Dr Lee Haines as acting secondary and tertiary supervisors.

Chapter 3- The design of the sample collection and development of the standard operating procedure for processing the samples were developed by the author in collaboration with Prof Steve Torr as his main supervisor. The training of field staff was carried out by the author and MSc Student Mercy Opiyo. Two technicians were trained, Victor Drapari and Edward Aziku, who between them carried out 95% of the tsetse collection, microscopy screening and dissection in the field. Mercy Opiyo processed ~5% of the tsetse samples in the field. The Processing of samples at LSTM was carried out by the author and Jessica Lingley, laboratory technician. The author carried out approximately ~55% of the tsetse fly analysis with the novel multiplex ITS primers and bloodmeal primers, including optimisation. Jessica Lingley carried out ~45% of the tsetse fly laboratory work.

Chapter 4- The design of this Chapter was a collaboration of the author with Prof Stephen Torr, Dr Emily Adams and Dr Lee Haines. The laboratory work was carried out by the author and Jessica Lingley and divided in a 60% to 40% split respectively.

Chapter 5- The design of the effects of tiny targets on local cattle was carried out prior to the author joining the Study. The design of the pig survey was conducted by the author under the advice of Prof Stephen Torr. The author joined the field work during the third sampling survey and joined two further sampling rounds. During this time the author participated in the work with Dr Inaki Tirados and Dr Johan Esterhuizen. The author carried out the pig sampling round with the help of local veterinary technicians and Henry Ombanya, the driver for the field work in Uganda. The laboratory work was carried out at the Liverpool School of Tropical Medicine by the author and Jessica Lingley each contributing 50% of total work done.

Data handling and Analysis- The data input for the different chapters was carried out by by the author, Dr Inaki Tirados, Dr Johan Esterhuizen, Victor Drapari, Edward Aziku and Jessica Lingley in equal measure. The data cleaning and primary analysis was carried out by the author, further analysis was carried out by the author and Prof Stephen Torr.

Abstract

Detection and control of *T. brucei s.l.* in the historic sleeping sickness foci of NW Uganda

Lucas J. Cunningham

Gambian human African trypanosomiasis (gHAT) is a deadly disease caused by *Trypanosoma brucei gambiense* transmitted by tsetse flies (*Glossina*) and is found only in West and Central Africa. The NW of Uganda has been known as a focus of gHAT since the early 20th Century but the number of new cases has reduced from 948 in 2000 to 4 in 2015. NW Uganda is therefore a good testing ground for alternative control and surveillance strategies that could be employed at other foci in the drive towards elimination.

Focussing on the Koboko foci of gHAT in NW Uganda, this study aimed to: (i) assess the prevalence of T. b. gambiense in the vector and potential reservoir host animals (cattle and pigs), (ii) develop a system for xenomonitoring gHAT and (iii) assess the impact of Tiny-Targets, a novel vector control technology, on the transmission of salivarian trypanosomes amongst local cattle.

The tsetse population was sampled continuously from April 2013-July 2014 using pyramidal traps (four traps operated on average 18 days/month). A total number of 12,532 *G. f. fuscipes* were captured. A subset of these tsetse (6,664) were analysed for the presence of trypanosomes using either microscopy and/or molecular methods. No tsetse tested were found to be positive for *T. b. gambiense* but *T. brucei s.l.* (2%), *T. vivax* (3%) and *T. congolense* (4%) were detected. PCR-based analyses of bloodfed tsetse (131) showed that the predominant hosts were humans (37%), cattle (39%) and Monitor lizards (15%). A xenomonitoring system based on commercially available loop-mediated isothermal amplification (LAMP) kits were tested for their suitability to detect *T. brucei s.l.* DNA in tsetse. The study demonstrated that the LAMP kits were highly sensitive, being able to detect the equivalent of 0.1 trypanosome/mL. They could also detect *T. brucei s.l.* DNA in tsetse midguts six days-post ingestion. Analyses of 2,877 cattle from areas where Tiny Targets were present or absent using microscopy and PCR-based methods found that the targets had no significant impact on trypanosome prevalence; prevalence of *Trypanosoma spp* in cattle from areas with or without targets was 10% for both sites. *T. b. gambiense* was not detected unequivocally in cattle or pigs.

This study showed that in a setting where gHAT is close to elimination it is extremely difficult to detect the parasite in the vector population. It also indicates that local cattle and pigs are not likely to be playing a role as reservoir hosts. The commercially available LAMP kits offer the basis of a novel and more cost-effective system for monitoring *T. brucei* parasites in low-prevalence settings.

Acronyms

AAT	Animal African Trypanosomiasis
ANOVA	Analysis of Variance
APOL1	ApolipoproteinL1
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CATT	Card Aglutination Test for Trypanosomiasis
CNS	Central Nervous System
CytB	Cytochrome B
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
FTA	Fitzco/Flinder Technology Agreement
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gHAT	Gambian Human African Trypanosomiasis
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Models
HAT	Human African Trypanosomiasis
IGS	Intergenic Spacers
ITS	Internal Transcribed Spacer
LAMP	Loop-mediated Isothermal Amplification
LOD	Limit of Detection
LSTM	Liverpool School of Tropical Medicine
LSU	Large-Subunit
mITS	Multiplex Internal Transcribed Spacer
NW	North West
OvC	Ovarian Category
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SRA	Serum Resistance Antigen

SSU	Small-Subunit
Tbr	*Trypanosoma brucei s.l.*
TgsGP	T. gambiense-Specific Glycoprotein
TLF	Trypano-Lytic Factors
ULV	Ultra-Low Volume
UV	Ultra Violet
VSG	Variant Surface Glycoprotein
WHO	World Health Organisation

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Chapter 1 : Introduction

1.1 History of sleeping sickness

1.1.3 Natural history of human African trypanosomiasis

Human African trypanosomiasis (HAT), commonly known as sleeping sickness, is a disease caused by species of *Trypanosoma*, single celled flagellated parasites from the order Trypanosomatidae. Within this family are other species of parasite that cause several important veterinary and medical diseases including animal African trypanosomisis (AAT), Chagas disease, also known as South American trypanosomiasis, and Leishmaniasis caused by the *Leishmania* parasite. Trypanosomes parasitize a wide range of hosts from plants (1), insects (2), fish (3), amphibians, reptiles (4), birds (5) and mammals (6). The life cycle of these parasites can either involve one host, a monogenetic life cycle, or two hosts, a digenetic life cycle (7). Earlier efforts to infer the evolutionary history of these parasites was limited to comparisons of life history and morphology (8). However, with the development of molecular biology it has been possible to study the phylogenetic relationships between the Trypanosomatidae (9). These studies have shown that the trypanosomes cluster into three separate clades: an African clade which includes the modern African trypanosomes; a second clade that incorporates extant South American and Australian trypanosomes and finally a third, aquatic clade. This suggests that the African trypanosomes evolved independently in Africa while the other trypanosomes evolved in an ancient Southern supercontinent (10).

During the course of the trypanosomes evolution two important events occurred one was the development of antigenic variation and the other was the parasitism of insects, which ultimately led to the close relationship between the salivarian trypanosomes and their insect vectors. Antigenic variation is a mechanism used by trypanosomes to evade the hosts immune system (11) by constantly changing the variant surface glycoproteins (VSGs) surrounding the trypanosomes when it is in the mammalian blood stream. This is essential as African trypanosomes live extracellularly and are therefore constantly exposed to the

host's immune system. In African trypanosomes, there are up to 2000 genes dedicated to VSG production, making up to 30% of the total genome. The combination of these genes allows for a near infinite number of new VSGs to be produced, meaning the trypanosome will always be able to present a new surface coat to the immune system (12). This constantly changing appearance of the parasite also means that, to date, no vaccines are available to protect individuals from this disease.

The second important evolutionary change was the ability to infect insects, which occurs in both monogenetic and digenetic trypanosome life cycles. It was suggested that as the monogenetic life cycle is simpler, involving one host and the digenetic life cycle more complex, involving two hosts, that it would follow the monogenetic life cycle was older. Trypanosomes with monogenetic life cycles have been described in insects, ticks and ciliates (7) and it was theorised that the African trypanosomes of humans and other mammals originated from parasites that had co-evolved with their invertebrate hosts (13, 14). The competing theory to this was that the ancestor of the trypanosomes originally colonised the gut of early aquatic vertebrates and evolved along with the vertebrate lineage, it later colonised the blood and from here was introduced to hematophagous insects, leading to the digenetic lifestyle of the modern vector transmitted trypanosomes (15-17). This uncertainty as to the origins of digenetic life cycle of trypanosomes was only resolved with advent of molecular phylogeny, which found that the earliest lineages to diverge were not the monogenetic trypanosomes but rather the digenetic parasites of mammals (18-21).

The importance of the digenetic lifecycle of the trypanosome parasites is that it has resulted in insect vectors which are capable of transmitting the disease which increases the basic reproduction number, R0, of the parasite (22). There are trypanosomes that are transmitted mechanically by vectors (e.g., *T. evansi* transmitted mechanically by horseflies) but in their case they cannot be counted as having a two host life cycle as there is no development within the vector. The major vector borne trypanosome diseases can be classified by the means of inoculation into the mammalian host. The salivarian group enter the host via mouthparts of the vector and are termed salivarian trypanosomes, while those parasites transmitted through defecation onto the host are classified as stercoraria (23). The veterinary and medically important salivarian African trypanosomes are transmitted by

members of the Dipteran order from the genus *Glossina*. The evolutionary history described above is reflected in the modern trypanosome diseases of humans and their domestic mammals.

1.1.2 Human origins and trypanosomiasis

The origins of the human species, *Homo sapiens*, some 200,000 years ago in Africa (24, 25) has resulted in the evolution of the *APOL1* gene which codes for the apolipoproteinL1 (APOL1) in humans and four other primates (26). The primate species that are known to possess trypano-lytic factors (TLF) are West African Guinea baboons (*Papio papio*) (27), mandrills (*Mandrillus sphinx*), gorillas (*Gorilla* gorilla) and the sooty mangabey (*Cercocebus* torquatus) . The four non-human primates with effective TLF are all considered terrestrial. This strongly implies that the more terrestrial an animal is the greater the selection pressure trypanosomiasis will play due to the increase in encounters with tsetse. The baboons show the greatest level of resistance of all the primate species being resistant to *T. brucei s.l.* (including the human pathogenic species)*, T. congolense s.l.* and *T. vivax* that commonly infect the local wild and domestic fauna (28).

1.1.3 Human trypanosome resistance

APOL1 is found in human serum and is associated with two different trypano-lytic factor complexes TLF-1 and TLF2 (29-31). These protect *Homo sapiens* from all forms of African trypanosomes except for two sub-species of *Trypanosoma brucei* that have evolved resistance to this innate immune response. Conversely the major domesticated mammals used in agriculture, cows, pigs, sheep, goats and horses originated from breeding stocks that evolved outside of Africa (32-36), this makes them susceptible to the trypanosomes of the local African ungulates, suids and bovids. This complex history of evolution and migration of people and animals in and out of Africa alongside the co-evolution between *Trypanosoma* and *Glossina* has resulted in the diseases of sleeping sickness and animal African trypanosomiasis (AAT), often referred to as nagana.

1.1.3 The study of African trypanosomes

Trypanosomes were first discovered in 1841 by Professor G. Valentin in the blood of a trout and later in that of frogs. Following this, other scientists made similar discoveries from descriptions in frogs and these works were published in 1842 and 1843 and it was within these early manuscripts that the name Trypanosoma first appeared. The naming of the parasite has been attributed to David Gruby and originates from the Greek 'trypano' meaning auger and 'soma' meaning body due to the undulating movement and sinusoidal appearance of the parasite. In subsequent years, trypanosomes were identified in a range of animals including the first mammals but it was not until 1880 that the link between disease and trypanosomes was made. This occurred in India when Griffith Evans was able to prove that trypanosomes were responsible for the disease surra in the local horse and camel populations. He did this by inoculating healthy animals with the blood of infected animals, these inoculated healthy animals then went on to develop surra, with observable trypanosomes in their blood (37).

Soon after this, in the early 1890s, British colonial farmers in South Africa were losing their herds of European cattle to a local veterinary disease named nagana, named after the Zulu word for low in spirit. To uncover the cause of this disease Surgeon-Captain and bacteriologist, David Bruce was dispatched to South Africa. By 1895 Bruce had described the trypanosomes from cattle suffering from nagana for the first time (38) and in 1903 identified the tsetse as the vector of the disease (39). In 1899 the parasite described by Bruce was named *Trypanosoma brucei* by Bradford and Plimmer after its discoverer. The first description of trypanosomes from a human patient in Africa occurred in 1901 when Michael Forde observed them in the blood of a boat captain operating in The Gambia, although they were only identified as trypanosomes by Joseph Dutton in 1902 (40). It was Dutton that proposed they name the human trypanosome *Trypanosoma gambiense* (41), this name has since been changed to *T. brucei gambiense* and is one of the two human forms of African trypanosomiasis. The second parasite responsible for HAT was discovered by Harold Fantham and John Stephens in 1910 when investigating the case of a 26 year old man from Northumberland who had contracted the disease in North-East Rhodesia (42). This second species of human trypanosome was originally named *T. rhodesiense* and this was later changed to *T. brucei rhodesiense.*

Between the discoveries of the two human forms of African trypanosomiasis other important discoveries had been made in relation to the vector and the implication of more than one species of animal trypanosome responsible for Nagana. Bruce had originally suggested that the transmission of trypanosomes between mammalian hosts by tsetse was a mechanical route (39) however Friedrich Kleine was able to demonstrate cyclic transmission of trypanosomes in *Glossina* (43). In light of Kleine's research Bruce would revisit his work and he was able to publish the complete life cycle within the vector (39). From 1904 to 1905 two new species of trypanosome were described by Alphonse Broden and Hans Ziemann. The first of these to be described was *T. congolense* (44) of which there are currently three recognised strains identified *T. congolense* Forest, *T. congolense* Savanna and *T. congolense* Kilifi (45-49). Ziemann identified *T. vivax* which is a genetically diverse species to the extent that it could be argued it is not a single species but rather these distinct genotypes are species in their own right (50-52). The combination of the two human species, *T. b. gambiense* and *T. b. rhodesiense* plus the three trypanosome species responsible for nagana, *T. b. brucei*, *T. congolense* and *T. vivax* make up the five major trypanosome species of veterinary and medical importance.

1.2 African trypanosomiasis

1.2.1 HAT

Human African Trypanosomiasis (HAT), often called sleeping sickness, is in fact two diseases caused by distinct sub-species of trypanosome , *T. b. gambiense,* which is responsible for 98% of all HAT cases in the last decade (53), and *T. b. rhodesiense*. These two parasite species have separately evolved a means to survive in the human blood stream, despite the presence of trypanosome lytic factors in human sera. Both forms of HAT are considered fatal if left untreated and have resulted in hundreds of thousands of deaths (54) although there is some evidence to indicate that HAT may not be 100% fatal (55). The two forms of HAT are clinically and geographically distinct with Gambian sleeping sickness being a chronic disease occurring in West and Central Africa and Rhodesian sleeping sickness, which is an acute disease, found in East Africa (Fig1.1). In both diseases there are two phases, an early haemolymphatic stage which is then followed by a second, neurological phase caused when

trypanosomes cross the blood-brain barrier and invade the central nervous system (CNS). It is this secondary phase that the name sleeping sickness derives from as the subsequent neurological damage caused by the parasites in the CNS affects the sleep-wake cycle of the human host.

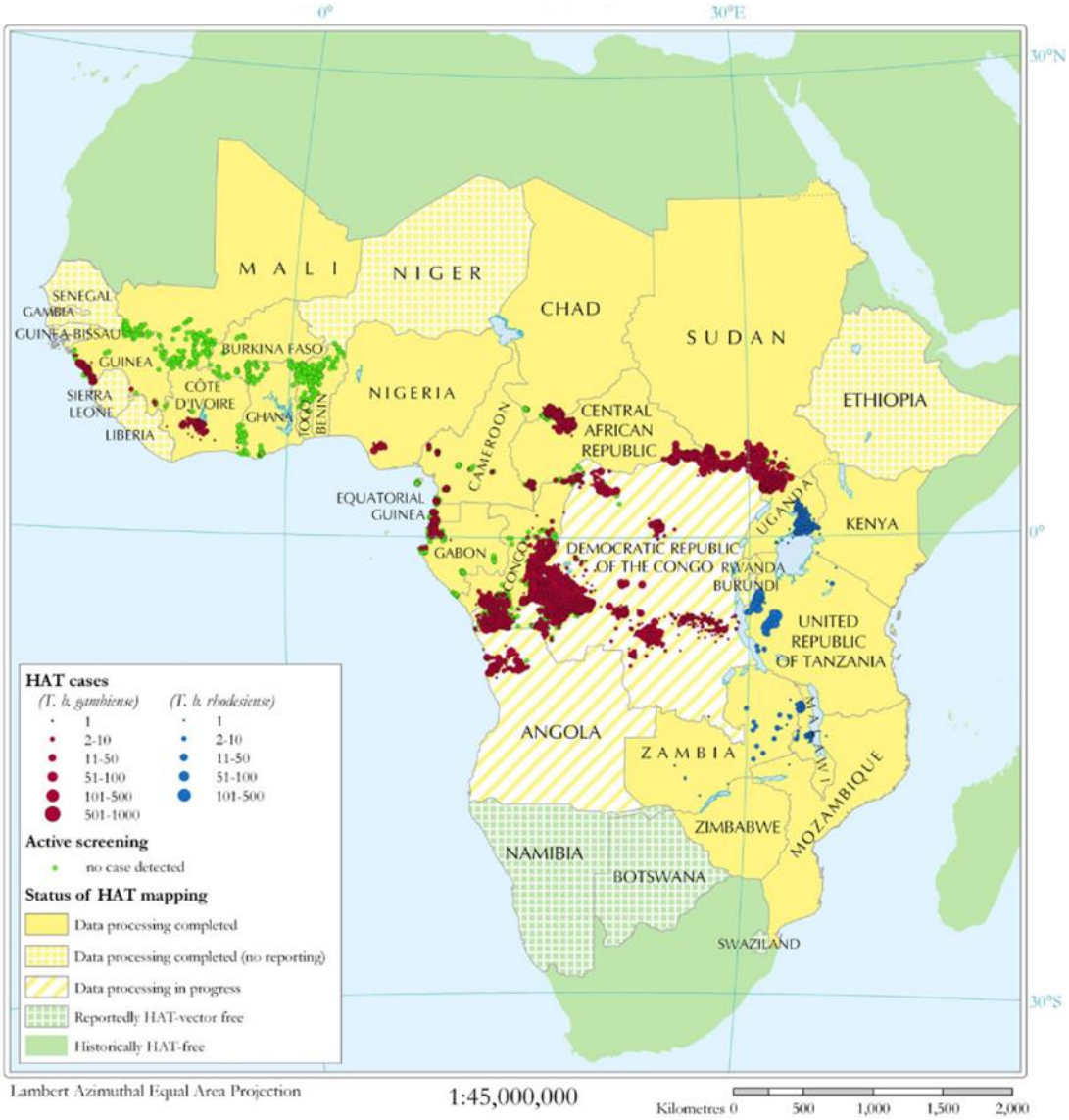


Figure 1.1 The distribution of T. b. gambiense and T. b. rhodesiense:
Image taken from The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases (56) covering the preriod from 2000-2009.

1.2.1.1 Trypanosoma brucei gambiense

It has long been recognised that it is many months – and sometimes years - before some people infected with *T. b. gambiense* starts showing clinical symptoms of disease (57, 58). This asymptomatic period of Gambian HAT varies, typically it lasts 18 months but the (54) longest recorded period between infection and the presentation of clinical symptoms was 29 years (59). Reports of long periods of asymptomatic cases of Gambiense sleeping sickness suggests the possibility that humans may be playing a role as reservoirs of disease in local sleeping sickness foci (60-62). However, the significance of these individuals in the transmission of Gambian sleeping sickness is not known. The role of animal reservoir hosts is also poorly understood (60, 63-65), although the general consensus is that Gambiense sleeping sickness is an anthroponotic disease which circulates amongst the human population via the tsetse fly (66). *T. b. gambiense* is described as having two genetically and phenotypically different groups within the one sub-species, group 1 is the most widespread and is always resistant to human sera, however group 2 has a variable resistance to human TLF (67, 68). Only in *T. b. gambiense* group 1 is the TLF resistance mechanism linked with the *T. gambiense*-specific glycoprotein (TgsGP) gene (69). This is a single copy gene that is unique to *T. b. gambiense* group 1, although TgsGP-like gene have been found in *T.b. brucei, T. b. rhodesiense* and *T. b. gambiense* group 2.(70). The mechanism behind this resistance is not fully understood but it is known that the TgsGP gene itself is essential in conferring resistance as reintroducing the gene into knockout lines of parasites restored resistance to human TLF (71).

1.2.1.2 Trypanosoma brucei rhodesiense

The clinical symptoms for *T. b. rhodesiense* manifest themselves a lot sooner than those of *T. b. gambiense* and death can occur within three months. Although it is classified as one of the two HAT species it is essentially a parasite of animals. It enters the human population when a person is inoculated by an infected tsetse. Once in the human population it can then spread between individuals, although this is rare outside of an epidemic. Therefore, unlike the Gambiense sleeping sickness the importance of domestic and wild animals in control efforts are given a high priority. There is also little evidence to suggest asymptomatic carriers of the disease in the human population.

1.2.1.3 Trypanosome lytic factor (TLF) resistance

Another difference between the two trypanosomes responsible for HAT is the mechanism each has for overcoming the TLF in human serum. Early studies had shown that the serum resistant phenotype would vary in *T. b. rhodesiense* when it is passaged through different host animals (72, 73). The changes in human serum resistance were due to changes in the selection pressure from the different host animals and resulted in a change in the VSGs expressed on the parasites surface (74). Analysis of the messenger RNA from both TLF-susceptible and TLF-resistant strains of *T. b. rhodesiense* allowed for the transcript responsible for the resistant phenotype to be identified (75-77). The transcript was coded from a VSG-like gene which has been named the serum resistance gene (SRA). Unlike the TgsGP gene in *T. b. gambiense* the SRA gene when inserted into *T. b. brucei* transferred complete TLF resistance (78). This was confirmed in vivo by the accidental self-inoculation by a researcher with the transgenic *T. b. brucei-SRA strain* (79). The TgsGP and SRA genes are considered unique for *T. b. gambiense* and *T. b rhodesiense* respectively and are used to distinguish the parasites responsible for HAT and differentiate them from each other and the animal trypanosome, *T. b. brucei*.

1.2.2 Treatment

1.2.2.1 Melarsoprol

Prior to the discovery of the transmission cycle of tsetse by Bruce, the tsetse fly had been suspected of causing disease in cattle but not in people, thus in parts of Africa local people would avoid taking their cattle to tsetse infested areas, or if they had to cross them they would do so at night (39). Once the discovery had been made that trypanosomes caused sleeping sickness and nagana, scientists worked to develop drugs that could treat the disease. One of the earliest drugs used to target trypanosomes was a preparation of arsenic called Atoxyl which had been synthesised by Pierre Bechamps in 1859 and was being used to treat sleeping sickness by 1905 (80). The Liverpool School of Tropical Medicine sent 2,800 grams of Atoxyl to Africa to be used in the treatment of individuals suffering from sleeping sickness in the early 20th century (81, 82). Despite being 40 to 50 times less toxic than arsenic a review of the drug by Robert Koch found that 2% of patients treated with Atoxyl were blinded by the drug due to atrophy of the optic nerve (83). Atoxyl was used to treat the haemolymphatic stages of sleeping sickness and in 1919 a derivative of Atoxyl that was

capable of crossing the blood brain barrier was developed (84). This new drug, tryparsamide, was used for treatment of the neurological phase of sleeping sickness, although it too was highly toxic and resulted in cases of blindness (83, 84). Another derivative of Atoxyl was developed by Ernst Friedheim, which was also highly toxic, but Friedheim combined this new drug, melarsen oxide, with the arsenic antidote, dimercaprol (85) to make melarsoprol.

Melarsoprol was introduced in 1949 for the treatment of second stage *T. b. rhodesiense* sleeping sickness. Although it showed no sign of toxicity toward the optic nerve (86) it can induce encephalopathy in 2-10% of patients of which 50-75% die due to this adverse side effect (87). Melarsoprol is still currently in use despite its toxicity.

1.2.2.2 Suramin

At the time Atoxyl was being assessed as a suitable candidate for the treatment of trypanosomiasis, the scientist Paul Ehrlich was assessing the chemotherapeutic potential of the compounds developed by the new synthetic dye industry in Germany. Ehrlich screened over 100 new synthetic dyes against mice infected with *T. b. brucei* and identified a compound called Nagana Red as having limited trypanocidal properties. It was thought that the limited effects of Nagana Red were due to its poor solubility in water and subsequent absorption into the bloodstream (83). To increase the effectiveness of the compound a version with improved solubility in water was developed called Trypan Red (80). Despite these improvements the new Trypan Red drug was only effective against *T. equinum*. In France a second dye Trypan Blue was later discovered to be very effective against trypanosomes by scientists Maurice Nicolle and Felix Mesnil (88, 89). The drawback of Trypan Blue was that it stained the patient so a colourless derivative of the compound was desired. Eventually the chemical Bayer 205, renamed Germanin, was discovered and was successful in clearing trypanosome infection in both animals and humans. Due to political complications the formula for the drug was withheld by the German company Bayer (90). Fortunately, a French pharmacist published the formula for Germanin and the drug was renamed Suramin and is still being used to treat the haemolymphatic stage of *T. b. rhodesiense* sleeping sickness.

1.2.2.3 Pentamidine

In developing a protocol for *in vitro* culturing of trypanosomes it was discovered that they require a large amount of sugar in their metabolism, consuming twice their mass in sugar every 24 hours (91). Following on from this discovery, investigations altering the blood glucose levels of infected animals were carried out (92) resulting in the identification of the oral anti-diabetic drug, synthalin, as a trypanocidal drug (93). Modifications of synthal by Arthur James resulted in two compounds with trypanocidal activities, stilbamidine and pentamidine (94). Currently only pentamidine is still in use for the treatment of the haemolymphatic stage *T. b. gambiense* sleeping sickness. Stilbamidine was dropped as a treatment and is no longer in use due to serious side effects (95, 96).

1.2.2.4 Eflornithine

The last drug to have been approved for treatment of HAT was the anti-cancer candidate drug eflornithine (97-99) which was first developed in the early 1970s (99). Almost a decade after its discovery, eflornithine was tested against trypanosomes in a mouse model resulting in complete cure of the mice from *T. b. brucei* (100, 101). Following on from the success of the use of eflornithine in the mouse models a number of trials treating human cases of *T. b. gambiense* were carried out (102, 103). These trial confirmed that eflornithine was an effective form of treatment for the neurological phase of *T. b. gambiense*, it was also effective against strains of *T. b. gambiense* that were showing resistance to suramin (104). Eflornithine has not proven an effective treatment against East African sleeping sickness due to *T. b. rhodesiense* having a higher turnover rate of the enzyme than the drug targets (105).

1.2.2.5 Current strategy and new developments

Since the approval of eflornithine for use as treatment for West African sleeping sickness, in 1990, no new drugs have been approved. A combination treatment of eflornithine and nifurtimox (a treatment for South American trypanosomiasis) was found to be as effective as treatment with elfornithine on its own (106). One of the most recent advances in the pursuit of a new treatment for sleeping sickness resulted in the identification of compound, GNF6702, which has cleared mice infected with *T. b. brucei*. This compound was also able to clear mice infected with other parasites related to the African trypanosomes, namely *T.*

cruzi and *Leishmania donovani* (107). Currently the screening and treatment of the human population is the main method of controlling West African sleeping sickness.

1.2.3 AAT

Animal African trypanosomiasis, or nagana, is the animal equivalent of HAT and is responsible for disease in many species of domestic animals It has been estimated to cost the economy of sub-Sahara an estimated $5 billion dollars per year, due to loss of animals and lower meat and milk yields in infected animals (108-110). The presence of the disease has also prevented the use of draft animals in agriculture and made large areas of Africa unsuitable for raising livestock and productive farming.

Like HAT, AAT is limited by the range of the vector and is confined to sub-Saharan Africa with the exception of *T. vivax* which has spread to South America (111) and is being transmitted by local tabanids (112). However, unlike HAT the trypanosome species responsible for AAT are found in both East and West Africa and are not as geographically discrete as *T. b. gambiense* or *T. b. rhodesiense.* There are three major species of trypanosomes that cause AAT, these are *T. brucei s.l., T. congolense* and *T. vivax*, (23) these parasites are able to infect most domestic animals (113). There are other species of trypanosome transmitted by tsetse that are of lesser or no veterinary importance, they are *T. simiae, T. grayi, T. godfreyi* and *T. suis/T. msubugwe*.

1.2.3.1 Trypanosoma brucei brucei

This parasite is the third member of the *T. brucei* group, the other two being the species responsible for HAT. *T. b. brucei* is found throughout the sub-Saharan region of Africa and is generally the least common out of the three main tsetse transmitted animal trypanosomes. There is no morphological difference between the three sub-species of *T. brucei s.l.* and the only way to distinguish the animal from the human species is to use molecular techniques to try and identify either the TgsGP gene or the SRA gene. However as these are single copy genes it is often not possible to identify the exact species of *T. brucei s.l.*. In the past inoculation of a parasite into a human volunteer was used to distinguish between the animal and human diseases (114). Closely related to *T. brucei s.l.* are two non-salivarian

trypanosomes, *T. evansi* and *T. equiperdum* (23). As neither of these trypanosomes are reliant on *Glossina* as an obligate vector they can be found across the globe and cause significant disease in mammalian livestock , although *T. equiperdum* is strictly a disease of equines. *T. evansi* is spread via mechanical transmission (115-117) causing the disease surra and *T. equiperdum* is a sexually transmited disease amongst equines and causes the disease dourine (23). The two trypanosomes responsible for *T. b. gambiense, T. b. rhodesiense* alongside the following animal trypanosomes *T. b. brucei, T. evansi* and *T. equiperdum* make up the five species that belong to the sub-genus *Trypanozoon*.

1.2.3.2 Trypanosoma congolense

T. congolense is one of the three most important trypanosomes responsible for AAT and is comprised of three different strains (23), *T. congolense* Savanna, *T. congolense* Forest and *T. congolense* Kilifi (118, 119). Of these three strains the most common are *T. congolense* Forest and *T. congolense* Savanna, with the latter being the most widespread across sub-Saharan Africa. The distribution of the different strains is dependent on the environment and the local vectors with the Savanna strain found in a wider range of *Glossina* species compared to the Forest strain which is restricted to *Glossina* species associated with riverine habitats (120, 121). All three strains of *T. congolense* belong to the subgenus *Nannomonas* to which there are three further species of trypanosomes: *T. godfreyi, T. simiae* and *T. simiae* Tsavo. These last three trypanosome species do cause nagana however they are not as prevalent as the *T. congolense* strains (118, 122).

1.2.3.3 Trypanosoma vivax

T. vivax are a genetically diverse group of trypanosomes (123-126) that are one of the major causes of nagana, especially in ruminants (23). It is also the only member of the medically or veterinary important species of salivarian African trypanosomes to have spread beyond Africa, despite the lack of any biological vector outside of Africa (111). The pathogenicity of *T. vivax* is dependent on the strain of parasite and on the vertebrate host (23). *T. vivax* has been assigned the subgenus *Dutonella*.

1.2.3.4 Trypanosoma theileri

As well as trypanosomes of veterinary and medical importance there are also many trypanosome species that appear to cause little or no pathology in the infected vertebrate hosts. One of the most common non-pathogenic trypanosomes is *T. theileri*, a trypanosome of cattle that is found on every continent except Antarctica (23, 127-132). Trypanosomes are classified as *T. theileri* based on morphological criteria including their large size (24µm-61µm) (133) and a small kinetoplast located near the nucleus. Another unique feature of *T. theileri* is its host specificity as it has been shown to be unable to infect non-cattle species of mammals (23, 134). Trypanosomes that are morphologically similar to *T. theileri* that are found in other mammal species are classified as *T. theileri*-like.

1.3 Identification of trypanosomes

1.3.1 Microscopy

Microscopy is the original method used by Bruce for the identification of African trypanosomes (38) and is still used to screen humans, animals and tsetse for the presence of a trypanosome infection. The identification of trypanosome species is complicated by the close morphological similarity between the different salivarian species, Fig.1.2, coupled with the fact that within a single species there can be a range of morphologies present in a single sample depending on the life cycle stage (135).

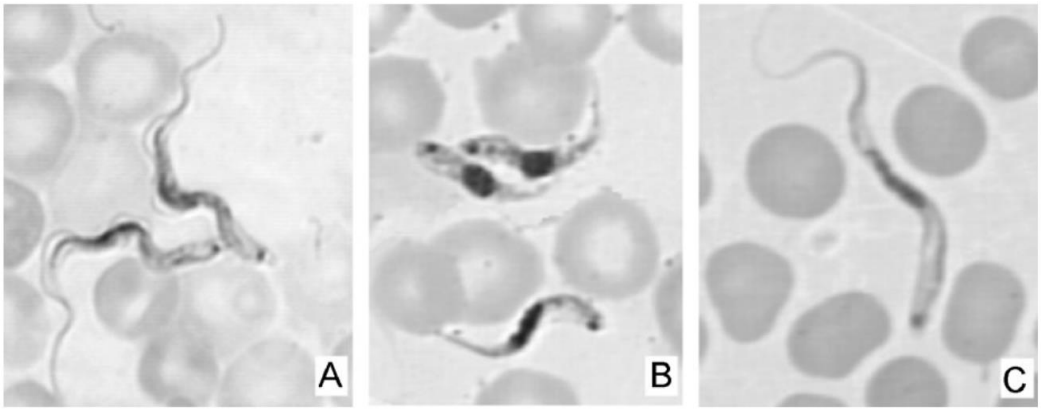


Figure 1.2 ***Images of three giemsa-stained trypanosome species:***
(A)*T. b. brucei,* (C)*T. congolense* and (C)*T. vivax*. Image taken from (136)

Diagnosis in humans is simplified by the fact that only two species of African trypanosome commonly infect humans and both these species are separated geographically. Therefore,

any presentation of trypanosomes in human blood in West and Central Africa would be designated as *T. b. gambiense* and likewise trypanosomes positive individuals in East Africa would be diagnosed with *T. b. rhodesiense*. The issue with this approach is that it relies on the current, discrete, geographical separation of the two HAT diseases, complications will arise if the two diseases ever overlap as the course of disease and treatment is different.

The identification of trypanosomes in the tsetse fly relies on the location within the vector for the three groups of salivarian trypanosomes, *Trypanozoon, Dutonella* and *Nanomonas*, as well as morphology. However not all species are morphologically distinguishable such as *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense* and unlike in the HAT parasites there are areas where these different species overlap. Similarly, trypanosomes that share the same developmental pathway are also hard to distinguish, such as *T. congolense* and *T. simiae*.

In other vertebrate hosts the same weaknesses associated with the identification in the vector and human hosts are also true, and with non-human vertebrate hosts there is also a greater range of species that can establish an infection. Therefore, the microscopy method is susceptible to lack of specificity. It also depends on the skill and training of the technician that is operating the microscope. A study checking the quality of microscopy diagnostics in the Democratic Republic of Congo (DRC) found that less than half of the diagnostic laboratories routinely screening for HAT were able to identify trypanosomes reliably in positive thin blood films (137).

1.3.2 Serology

Typically, serology relies on the identification of specific antibodies that have been generated in response to particular pathogen or foreign proteins (antigens). In 1978 a new diagnostic assay for *T. b. gambiense* sleeping sickness was developed called the card agglutination test for trypanosomiasis (CATT) (138). This method uses a suspension of latex beads that are covered in one of the predominant VSG proteins expressed by *T. b. gambiense* to act as an antigen to screen for the presence of specific anti-bodies in the patients serum.. When either the serum or blood of an infected individual is added to the latex bead suspension the beads and antibodies adhere to each other via the antigen on the beads surface, causing a clumping together of the two. This is visible to the technician

performing the assay and is the indication of a positive result. A negative serum or blood sample would not produce this clumping effect and the test remains even in appearance. The CATT test is specific, it does not require a high degree of technician training and it is cheap and quick to carry out making it useful for large scale screening of the human population. The negative aspects of this diagnostic method is that despite its good specificity it has a poor positive predictive value, requiring those that have tested positive with the CATT test to undergo further parasitological examination (139). Apart from the CATT test there are currently no other field friendly serological diagnostic test for the other trypanosome species although a 2nd generation RDT designed to detect HAT is under development (140). In order to develop serological diagnostic assays for the other species of trypanosomes antibodies specific to these parasites need to be raised and then checked for cross reactivity with similar species.

1.3.3 Molecular methods

Molecular methods can be used to identify differences in the genomes of organisms as opposed to relying on morphological differences, which in the case of the salivarian trypanosomes are often limited. Another advantage of using DNA to distinguish trypanosome species is that unlike the morphology it does not change depending on the life stage of the parasite. The first molecular methods used to identify trypanosome species were based on DNA hybridisation probes (141). These were short sections of satellite DNA repeats that were specific for the intended species of trypanosomes. These non-coding repetitive DNA elements were used as they often had high copy numbers, which increased the sensitivity of the test and were poorly conserved between species increasing their specificity (142). These early assays were able to detect low levels of trypanosomes and distinguish between the morphologically similar species, including the three different strains of *T. congolense*, something that could not be done with microscopy methods (142).

1.3.3.1 PCR

The discovery of the polymerase chain reaction (PCR) in 1983 credited to Kary Mullis (143) superseded the DNA hybridisation probe method due to the copying of the target sequence. PCR uses two complementary short codes of DNA, called primers, to target a specific region

of the genome for amplification. One primer is upstream of the region of interest and the other is downstream. The temperature of the reaction is increased to the point at which the double stranded DNA is denatured and separates, as the reaction cools the primers are able to attach to their sequence on either one of the single strands of DNA. The complimentary strand of DNA is then generated when a DNA polymerase extends the primer sequence. This process is repeated each cycle so in 30 cycles, if the amplification is 100% efficient, a single copy of a double stranded DNA sequence will result in over 100 million copies.

To visualise the results, the PCR the product is run out on a gel in a process called gel electrophoresis. The theory behind this is that the target of the primers will be a specific number of base pairs (bp) and different length products will move through a gel matrix at different speeds with smaller products moving faster than larger ones. The PCR product is added to a well at one end of the gel and an electric current is passed through the gel. DNA being negatively charged travels through the gel towards the anode, and it is during this process that any PCR product present in the gel gets separated by size. To gauge the size of the products, a molecular weight marker is run through the gel at the same time. The products produced during the PCR reaction are dependent on how well the reaction has been optimised and on the design of the primers for the purpose of the experiment. The specificity of the primers are dependent on the purpose of the assay. Some PCRs are designed to be highly specific, targeting only a single species, while others can be more generic, resulting in the amplification of DNA from multiple species. The choice of using either species specific or generic primers is part of the experimental design.

1.3.3.2 Species specific primers

These primers target variable regions within the parasites genome that are unique to the species of interest. The major salivarian species of trypanosome all have their own species specific primers, with the exception of *T. b. brucei* which cannot be positively identified as such due to a single gene difference between it and *T. b. rhodesiense*. Although being highly specific the disadvantage of species specific primers is that multiple reactions are required to screen a single sample for multiple trypanosome species resulting in an increase in cost. Another limitation of species specific primers is that they are unable to identify novel trypanosome species.

1.3.3.3 Generic primers

Generic primers are designed to target conserved regions of the genome that flank a variable region. The conserved regions allow for a wide range of trypanosome species to be targeted while the variable region allows for species identification. There are several ways that the different species can be identified, the variable region between the two conserved sites can be of different lengths resulting in different sized PCR products for each species. These can then be separated out using gel electrophoresis and sized by using the molecular weight markers. A common region of the genome that has been targeted for development of generic primers for trypanosome is the spacer DNA between the small-subunit (SSU) and the large-subunit (LSU) ribosomal RNA (rRNA) genes. These are referred to as internal transcribed spacer (ITS) regions and in eukaryotes there are two ITS regions, ITS1 and ITS2, separated by the 5.8S rRNA gene. These rRNA and ITS genes occur in multiple copies separated by non-transcribed DNA named intergenic spacers (IGS) Fig 1.3.

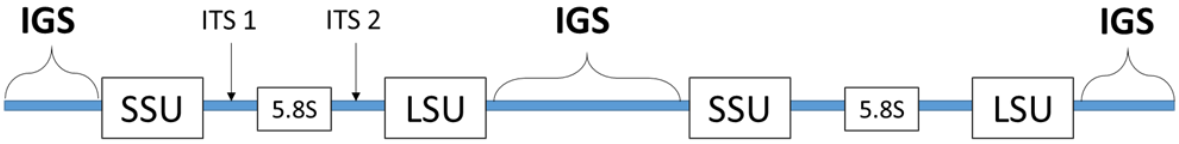


Figure 1.3 ***Diagrammatic representation of the positions of the ITS 1 and ITS 2 regions:*** in relation to the neighbouring genomic regions, image adapted from (144).

ITS 1 and ITS 2 have become popular targets for phylogenetic and taxonomic studies as they have a high copy number and they are highly variable. There are several generic primer assays that have been designed to identify trypanosome species based on the size of the PCR product generated (145-147). These generic PCRs are advantageous as they can identify a wide range of trypanosome species in the same reaction, including novel species.

1.3.3.4 Restriction fragment length polymorphism (RFLP)

Another method that has been used to identify different trypanosome species is the use of PCR restriction fragment length polymorphisms (RFLP). This method uses PCR to amplify the 18S variable region with the SSU gene, this stretch of DNA is then cut by restriction enzymes resulting in multiple fragments of varying sizes. When run out on a gel these different size fragments produce a banding pattern (148). This technique was able to distinguish all of the different salivarian trypanosome species transmitted by tsetse, however this method is less

accurate with multiple infections as some species share similar banding patterns, notably *T. congolense* and the non-salivarian species *T. theileri* (149).

1.3.3.5 Sequencing

Alternatively, if it is not possible to distinguish the different species based on the size of the PCR product, they can be sequenced to determine the exact genetic sequence of a product. Sequencing has the advantage of being able to compare your sequence to published sequences available on online databases like GenBank (www.ncbi.nlm.nih.gov). Using bioinformatics software, it is possible to compare sequences generated by one study to those of other studies, this is especially useful if the generic primers amplify an unknown trypanosome species. Even if it has never been described before it would be possible to observe its phylogenetic relationship with the known species. The negative side to sequencing is that it adds an extra cost to the processing of samples and complications arise when there are mixed infections within one sample.

1.3.3.5 Loop-mediated isothermal amplification (LAMP)

An alternative molecular method to PCR is loop-mediated isothermal amplification (LAMP) developed at the start of the 21st century (150). Like PCR, LAMP is capable of replicating a specific DNA target, however unlike PCR LAMP uses up to six different primers and an auto-cycling strand displacement *Bst* DNA polymerase. The six different primers are designed to target different regions of the replicating DNA resulting in a branching loop structure that is capable of continual amplification by the *Bst* DNA polymerase without the need for multiple heat steps. This isothermal property of the method means that an expensive thermocycler is not required for the reaction and instead a simple heat block is sufficient. The amount of DNA that is amplified using this method is enough for the turbidity of the reaction mix to increase when the target DNA is present, and it is this change that indicates a positive or negative result. This does away with the need to run the reaction out on a gel to determine the results and the addition of a fluorescent dye increases the sensitivity of detecting this changes in turbidity (151).

A LAMP assay targeting the *Trypanozoon* sub-genus was developed in 2007 and the results showed that the assay was both highly sensitive and reproducible. The simplicity of detecting the results by checking for turbidity in the reaction mix and the simplicity of the

sample preparation makes this method a good candidate for use in a clinical setting (152). Subsequent investigations further developed the reliability of the visual detection system (153), demonstrated the superior sensitivity of the assay to PCR (154) and improved the clinical applicability of the assay by developing lyophilised kits and simplifying the sample preparation (155).

1.4 The vector of African trypanosomaisis, *Glossina s.l.*

1.4.1 Forests, rivers and savannas

The biological vector of the African salivarian trypanosomes are members of the genus *Glossina*; these flies are commonly called tsetse. There are a total of 22 *Glossina* species that include five sub-species complexes (156) found discontinuously across ~10 million km^2 of sub-Saharan Africa (157). Tsetse are limited to Africa due to the Sahara desert to the North although a small population from Saudi Arabia was reported in 1910 (158). The limited range of the vector has prevented the spread of HAT and AAT beyond Africa, with the exception of *T. vivax,* which is currently being spread by mechanical vectors in South America. The tsetse species have been organised into three groups based on their external genitalia (Fig1.4), these are *Morsitans (Glossina), Palpalis (Nemorhina)* and *Fusca (Austenina)*.

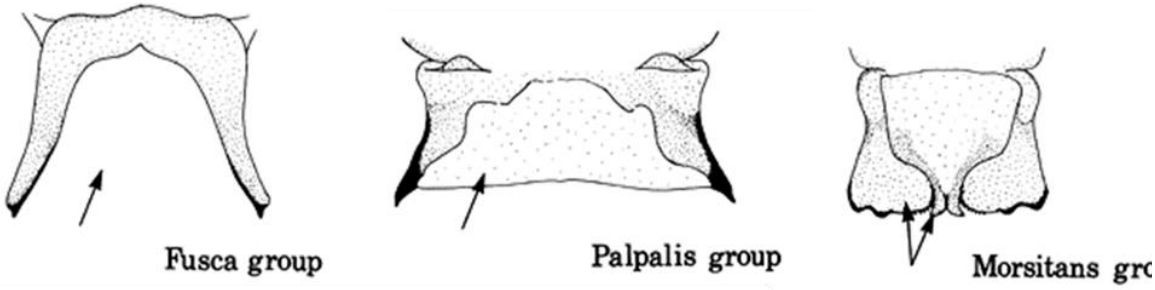


Figure 1.4 ***Illustrations of the different morphologies of male superior claspers.***
The claspers of the *Fusca* male lack a membrane and end in claws, the *Palpalis* male's claspers have a membrane and the claws are narrow. The *Morsitans* claspers are more specialised and no longer poses claws. Image taken from the FAO training manual for tsetse control personnel (159).

The *Palpalis, Morsitans* and *Fusca* groups correlate roughly with the favoured habitats that the different groups can be found in. These three types of habitat are forest, savanna and riverine environments, the corresponding tsetse groups are *Fusca, Morsitans* and *Palpalis* (160). The correlation between genital morphology and habitat reflects the evolution of the

different tsetse species. The *Fusca* tsetse have the most primitive genital morphology, followed by the *Palpalis* group and the *Morsitans* are considered the most recently evolved (161). It follows then that the original environment was a forest habitat, from here the tsetse moved out to riverine and then savanna habitats, this adaptation to increasingly arid environments can also be seen in the ability of tsetse pupae to resist dessication (162). Of the 22 species of tsetse only 11 are known to transmit sleeping sickness, six from the *palpalis* group, five from the *morsitans* group and 0 from the *fusca* group, (Table 1.1).

Table 1-1 Glossina *species that are known to transmit* HAT *(163)*

Palpalis	***Morsitans***
Glossina palpalis -gambiense	*Glossina morsitans centralis*
Glossina palpalis palpalis	*Glossina morsitans morsitans*
Glossina tachinoides	*Glossina morsitans submorsitans*
Glossina fuscipes fuscipes	*Glossina Swynnertoni*
Glossina fuscipes quanzensis	*Glossina pallidipes*
Glossina fuscipes martinii	

The ecological niches that the different tsetse species occupy determines their geographical distribution, the *Fusca* and *Palpalis* groups are mainly found in West and central Africa whilst the *Morsitans* tsetse can be found mainly in East Africa (160) Fig1.5.

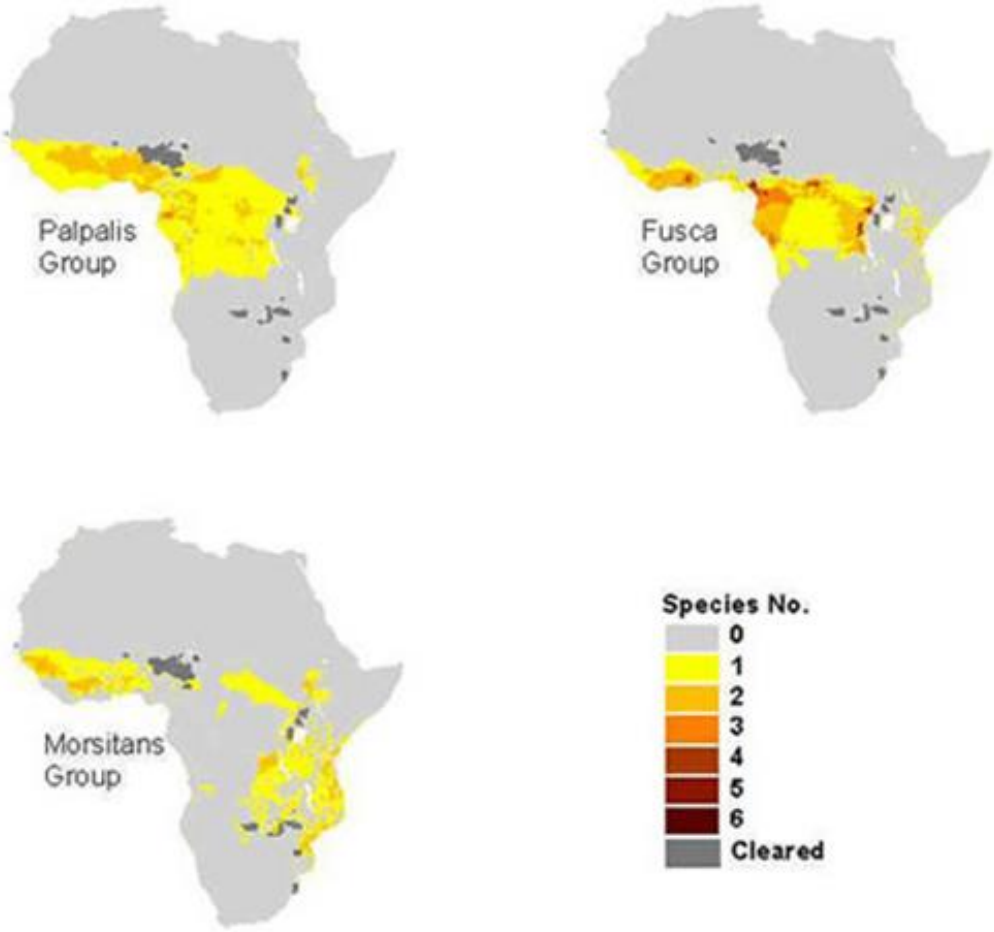


Figure 1.5 ***Distribution of the different tsetse groups across Africa:***
showing the concentration of Palapalis and Fusca species in West Africa and the Morsitans tsetse in East Africa. Image taken from medical ecology website (164).

1.4.2 Host seeking

The geographical distribution of tsetse species is also reflected in the vectors responsible for the transmission of the two types of HAT with the *Palpalis* group transmitting *T. b. gambiense* and the *Morsitans* group transmitting *T. b. rhodesiense*.

The habitat a tsetse lives in influences the host seeking strategies that tsetse employ. Morsitans tsetse, typically found in savanna woodland, locate their hosts using a combination of visual and olfactory cues, particularly the latter (165, 166). Host odours include volatiles such as 1-octen-3-ol, ketones, acetone and products of respiration such as carbon dioxide (165-167). Tsetse use olfactory cues to locate the host from long range by flying upwind of the odour plume and visual cues to locate and land on the host at closer proximity (168, 169). In contrast, Palpalis group tsetse, typically found in riverine habitats, are less responsive to host odours and rely more on visual cues (170, 171).

Palpalis and *Morsitans* tsetse are attracted to different sized hosts with the savanna tsetse preferring larger hosts such as bush pig and kudu while the riverine tsetse are more attracted to smaller hosts such as monitor lizard (172). It has been hypothesised that these differences in behaviour can be attributed to the geometry of the habitat, whereby barriers to tsetse movement and host location could be formed from dense bushes (173) thus necessitating the tsetse to adopt different feeding patterns. The importance of understanding the interaction between the visual and chemical signals given off by the host is essential in designing effective control technologies that can mimic these to the point of passing as a valid target for hungry tsetse (171). The third group of flies, fusca tends to be considered of minor importance in the transmission of African trypanosomiasis as they are not recorded as feeding on humans and are only ever found in heavily shaded bush where cattle are rarely encountered (174, 175) although there has been some attempt to re-evaluate their role as a vector(176).

1.4.2.1 Blood feeding

The tsetse fly belongs to the superfamily *Hippoboscoidea*, all members of this family, both male and female, are obligate blood feeders and have been recorded feeding on birds, mammals and reptiles (177). Tsetse have previously been described as opportunistic feeders, however this is generally only true when selective pressure is placed on the tsetse through the reduction of host diversity (172, 178). A recent study by Harriet Auty, that compared identification of tsetse blood meals with host density, has confirmed that Morsitans-group tsetse are highly selective in their feeding (179). Analyses of the diet of Palpalis-group tsetse suggests that they are less selective (172, 178).The identification of the host species that tsetse fed upon provides a better understanding of the transmission dynamics, especially with regards to reservoir hosts. It also provides the opportunity for intervention, such as treating the preferred domestic animal host with insecticide (180-182).

1.4.3 Blood meal analysis

1.4.3.1 Direct observation of feeding

The earliest and simplest method that was used to try and identify the origins of blood meals is to try and observe feeding behaviours of tsetse in the field. Directly observing tsetse feeding is difficult due to their small size and the short amount of time they spend feeding on the vertebrate host. This method is generally unreliable and is prone to bias based on what the researcher is able to track and observe and without extensive surveys it would be hard to determine the value of results generated (183-185). Although this method can be improved by having a range of bait animals to test the attractiveness of different hosts to tsetse (186) but even in this instance the presence of humans near bait animals has been shown to create a bias within experiments(187), although this can be negated with the use of electrical screens that catch flies as they approach and leave the hosts (188).

1.4.3.2 Red cell morphology

The analysis of fresh blood from the midgut of tsetse to determine the origin of the blood meal based on morphological features of the blood, such as nucleated erythrocytes found in birds and reptiles, (189) was used in the past. However this method lacks specificity and the morphology of erythrocytes can vary. The size of cells can be affected by digestive processes within the insect gut and there is often a range of cell sizes within the same species depending on the age of both the cell and animal (190).

1.4.3.3 Serology

It was in 1914 when the use of serology to identify the origins of blood meals in blood-sucking invertebrates was first demonstrated (190) and by 1963 a massive collection of over 22,000 tsetse blood meals from 15 species of tsetse spanning the years 1953-1962 had been collected and analysed using serological precipitation for 33 species groups(191). A larger serological study using an enzyme-linked immunosorbent assay (ELISA) to screen 29, 245 flies confirmed previous findings (172). Serological methods of blood meal identification were a vast improvement on visual field observations and could even detect blood meals three days post feed(192). This method is limited when trying to increase the resolution down to the species level as more tests need to be conducted, and in order to identify each species a specific antiserum needs to be developed.

1.4.3.4 Polymerase chain reaction (PCR)

PCR allows for the identification of blood meals to a far greater level of detail and with less laboratory work. Similar to the identification of trypanosome species, a number of molecular methods for the identification of blood meals have been developed, including RFLP methods targeting the hosts mitochondrial DNA (193-195). Allowing for multiple hosts to be identified with a single primer pair and restriction enzymes (196, 197). There are also multiplex PCR assays that combine multiple sets of species specific primers that each produce their own uniquely sized PCR product (198).

An alternative method to the species specific primers are the generic primers that target conserved regions flanking areas of variability and rather than use product size or enzyme digestion to identify the organism, sequencing is used instead (199). This has the advantage of not needing to know what potential hosts are in the environment and if any unknown animal is detected it would be possible to identify it easily to the genus level due to the high number of DNA sequences of known animals available online from various databases.

1.4.3 Trypanosome development in tsetse

The tsetse fly, as a vector of the salivarian trypanosomes is also a victim of trypanosomiasis itself and as such they have evolved their own immunological defence (200, 201) and a physical barrier that surrounds the blood meal to protect the tsetse from blood borne pathogens (202). The majority of tsetse in a population are refractory to trypanosomes and this is evident in the low rates of mature infections found in wild tsetse (203). The age of an individual tsetse is an important contributor to its susceptibility to infection. A newly emerged tsetse that has not taken a blood meal is termed a teneral fly. These teneral tsetse are highly susceptible to infection due to a reduced immunological competency (204) and the peritrophic matrix (PM) is not yet fully formed (205). The PM is a protective sheath that lines the gut of the tsetse and serves as a protective barrier for the tsetse. The importance of the midgut environment is probably greater for both *T. brucei s.l.* and *T. congolense* than it is for *T. vivax* as they have different developmental cycles within the vector host.

Both *T. brucei s.l.* and *T. congolense* establish an immature infection in the midgut, they then develop mature infections in the salivary glands and mouth parts respectively. *T. vivax* carries out its entire developmental cycle in the mouth parts of the tsetse(135) (Fig1.6).

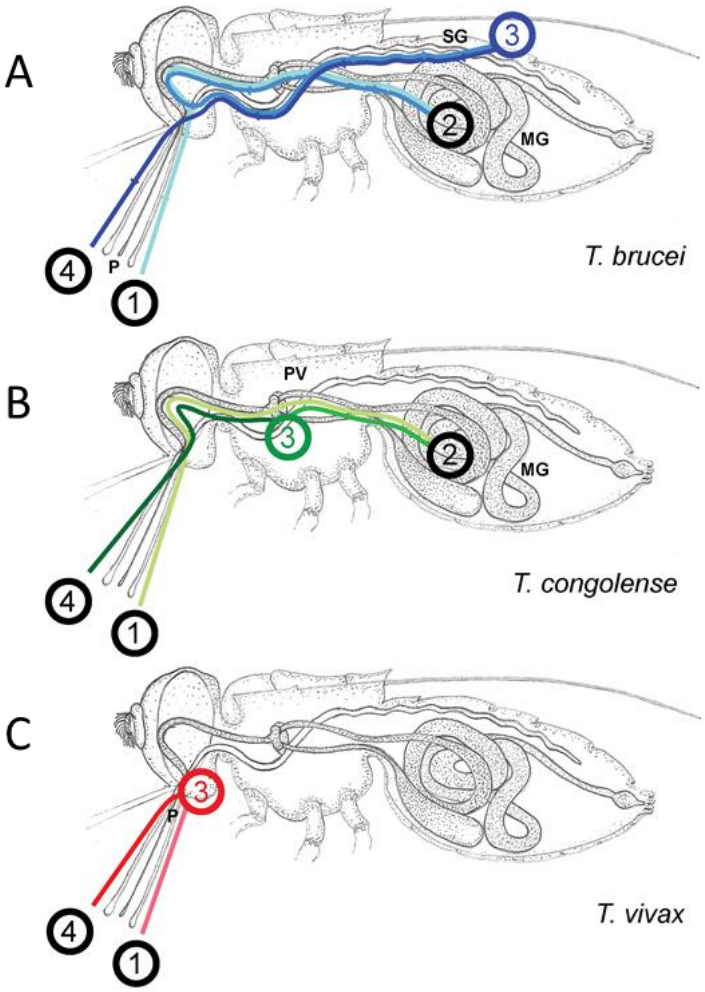


Figure 1.6 ***Illustrations of the different developmental routes for salivarian trypanosomes***
The development of African salivarian trypanosomes, within the vector, varies depending on the species of parasite. The three most important trypanosome species from both a veterinary and medical stand-point are T. brucei (A), T. congolense (B) and T. vivax (C). The figure divides the developmental cycle into four stages, ingestion of blood-stream form parasites (1); the passage of trypanosomes to the midgut (MG) of the tsetse and subsequent development into procyclic form (this step is absent in T. vivax development); anterior migration of trypanosomes and differentiation into epimastigotes in the proventriculus (PV) (T. congolense) or salivary glands (SG) (T. brucei); the final stage is inoculation of metacyclic forms into a vertebrate host during feeding (4). Image taken from Jackson 2015 (206).

1.4.4 Reproductive cycle

The *Hippoboscoidae* share a reproductive cycle that is characteristic of their superfamily and plays an important role in the susceptibility of tsetse to vector control. The tsetse fly differs from the other major vectors of medical and veterinary diseases in that they do not lay eggs but rather give birth to a single 3rd instar larva every nine days with the mother providing all the nutrition required by the larva while it grows within the mother and for subsequent pupal development. This large maternal investment and low rate of reproduction – an

example of a *K*-selected reproductive strategy (207) – contrasts with the much higher rates of reproduction and r-selected reproductive strategies displayed by most other vectors. The larva is nourished by 'milk glands' within the tsetse uterus that produces a milk-like substance which the growing larvae feeds from. It normally takes 9-10 days for the complete development of the 3rd instar larvae in the uterus of the tsetse, after which the larva is deposited on loose sandy soil. Having been deposited the larva burrows into the ground where it then pupates to form a puparium. The developmental time within the puparium for the larvae to transform into an adult tsetse is dependent on temperature; at 24°C it takes 30 days for the adult tsetse to emerge. Whilst ensconced underground the developing tsetse is relatively safe from predators and inclement weather, barring flooding the puparium remains safe. Once fully developed the young tsetse emerges from the puparium and burrows to the surface using its ptilinum, a sac like structure that when pumped expands and contracts helping the tsetse move through the soil. Following emergence from the soil females are fully fertile but males will take three days to fully mature (208). This reproductive cycle is represented diagrammatically in Fig.1.7.

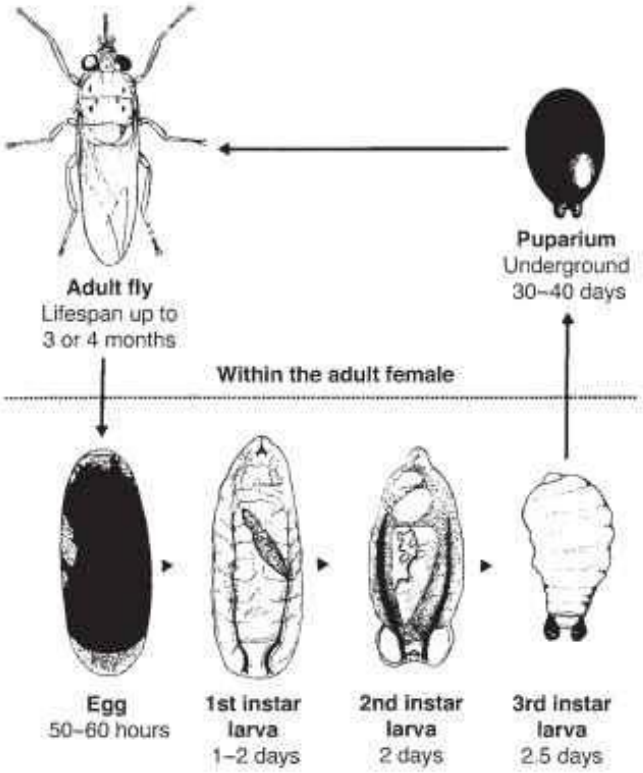


Figure 1.7 ***Diagrammatic representation of the life cycle of the tsetse fly***
Image taken from Tsetse Biology and Ecology: Their Role in the Epidemiology of Trypanosomiasis (161).

1.4.5 Tsetse control

One of the earliest tsetse control efforts was successfully conducted on Principe Island off the coast of West Africa in the Gulf of Guinea. This early campaign against *Glossina palpalis* was successful in eradicating the vector from the island through a combination of methods that would become the blue print for sleeping sickness control Across Africa during the early and mid-20th century. The methods employed to control the tsetse included bush clearing, the extermination of wild animal hosts and the use of a sticky trap to catch adult tsetse (82).

1.4.5.1 Bush clearing

The rationale behind bush clearing was that tsetse are known to favour different habitat types and the destruction of these habitats makes the area unsuitable for tsetse. There were two forms of bush clearance selective and sheer clearing. In the case of selective clearing it was found to be not as effective as the wholesale destruction of the tsetse habitat depending on the species of tsetse involved (Nash 1940, Harley and Pilson 1961). Due to the ecologically destructive nature of bush clearing it has fallen out of favour and is no longer used as a method of tsetse control.

1.4.5.2 Animal destruction

The destruction of wild host animals was carried out on the island of Principe and later would be carried out on a large scale after the observation was made that tsetse numbers correlated with those of wild animals (Da costa 1916, Jack 1914). Initially this involved the destruction of all large wild mammalian species but following the development of blood meal identification techniques are more selective approach was used. By destroying the wild hosts the intention was to starve the tsetse by limiting their likelihood of finding a blood meal. Despite its obvious success this strategy became increasingly unacceptable especially as general recognition of the value of biodiversity emerged in the 20th Century. Moreover, the long-term value of this approach became less obvious as it became clear tsetse would switch their feeding from wild animals to domestic ones, increasing the risk of nagana and East African sleeping sickness being introduced to the local communities. Despite both habitat and wild animal destruction no longer being an intentional method of tsetse control the encroachment of the human population into wilderness areas means that this process still impacts on tsetse populations.

1.4.5.3 Traps

The use of traps or targets for tsetse control can be traced back as early as 1906 when Senhor Bulhões Maldonado invented the sticky traps worn by the workers on Principe island (Fig.1.8.A). Over the course of 21 months, from April 1906 to the end of 1907, 133,778 tsetse were caught by this method on the Maldano estate. It was observed that two men wearing a sticky trap each whilst working in the plantation caught 1,500 – 2,000 tsetse at the start of the week and by the end of the week this number had dropped to 20 (82).

The success of the sticky traps on the island of Principe was enhanced by the reduction of alternative hosts through the destruction of local pigs, dogs and civet cats.
Screens had been used to attract tsetse by Swynnerton since 1922 (Fig.1.8.B) and following the development in 1930 of a trap for *Stomoxys* attempts were made to turn the screens into traps with the addition of a "non-return" wire cage(Fig.1.8.C) (168).
Later in the 1930s a trap was developed by Harris that was used to control the tsetse population in Zululand (Fig.1.8.D). From 1931 to 1938 it is estimated that over 10.5 million tsetse were caught using the Harris trap, resulting in the reduction of nagana in the local cattle populations (209). Harris traps were used in a variety locations such as in the Congo where 12 traps reportedly reduced the number of new HAT cases from 20 to 0 (161). Despite the success of the Harris trap it was not widely deployed, this could be due to the fact that it did not perform equally well with all tsetse species (168).

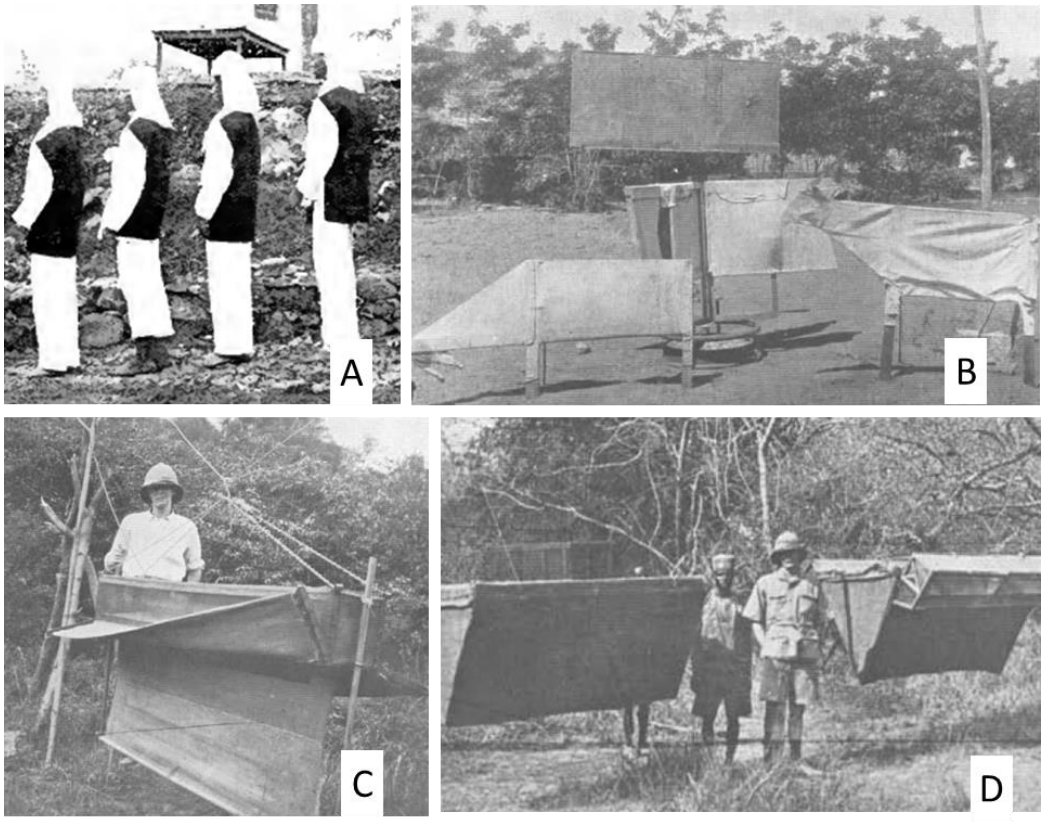


Figure 1.8 ***Images of early tsetse traps and targets:***
Sticky targets used for the control of tsetse on the island of Principe (A). The original targets used by Swynnerton, including plain oblongs as well as animal shaped targets (B), Swynnerton's target with non-return cage attached (C) and the Harris traps (D). Images taken from (82) and (168)

Different tsetse trap designs were developed throughout the 20th century with the development of the biconical trap (Fig.1.9.A) by Challier and Lavissiere (210, 211) The biconical trap was further developed into the Lancien trap (Fig.1.9.B) (212), pyramidal trap (Fig.1.9.C) (213) and the Vavoua trap (Fig.1.9.D) (214). The biconical trap and its variants were developed to target the *Palpalis* group of tsetse in West and Central African.

Figure 1.9 ***Images showing modern tsetse traps derived from the biconical design:***
(A) The original biconical trap and the three traps that subsequently derived from it, (B) the Lancien trap, (C) pyramidal trap and (D) Vavoua trap. Images taken from (215).

Trap development designed to target the *Morsitans* group of tsetse was also being developed during this period. In Zimbabwe the F3 trap was developed, this design was improved to make it easier to setup, resulting in the epsilon trap (Fig.1.10.A) (216). The F3 and epsilon traps were again re-designed to produce the M1, M2 and M3 series of traps of which the M3 performed the best (217). Other traps designed for savanna tsetse include the H-trap (Fig.1.10.B) (218) and NGU trap (Fig.1.10.C) (219, 220).

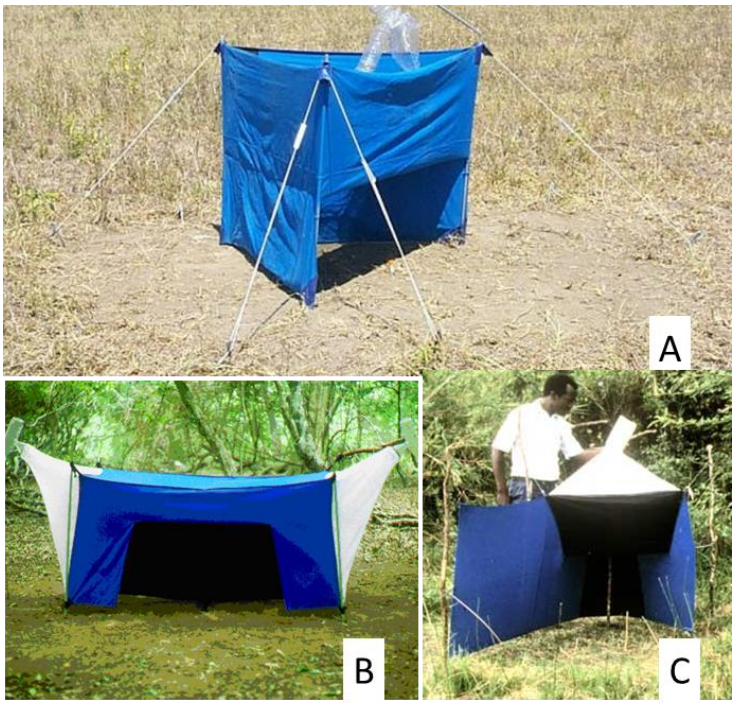


Figure 1.10 ***Traps designed to catch the savanna tsetse:***
(A) the epsilon trap, (B) H-trap and (C) NGU trap, images taken from (215)

The design of the traps work by taking advantage of the tsetse's attraction to particular wavelengths of light (221) in particular to combinations of black and a specific shade of blue called phthalogen blue (221, 222). Alongside the visual cues artificial odours have been developed that mimic those of a host animal. These artificial odour baits mimicked breath, urine (165, 223, 224) and skin secretions (225). These odours do not work equally well amongst the different tsetse groups as they are very effective in attracting the *Morsitans* tsetse but have little effect on the *Palpalis* group (161).

The deployment of traps can be used purely for control or they can be used as a method of collecting live tsetse from the wild populations for study. If the traps are being used as a control device the lethality of the trap can be enhanced by the application of insecticides (226, 227). Another form of control that developed in parallel with the trap technology is the development of the targets or screens. This method of control works in a similar way to the traps in that the targets attract the tsetse primarily through visual cues based on a contrasting blue and black pattern. However whereas the traps can still operate without insecticide, albeit with a lower lethality, the targets killing power comes from being impregnated with a fast acting contact insecticide (227-229).

1.4.5.4 Targets

The combination of visual and odour attractants is more effective against the savanna group of tsetse, requiring four targets per km^2 (166) in order to control the population. Riverine tsetse are less attracted to the artificial odours and the visual targets developed by Laveissière (230) resulting in a far higher number of targets needing to be deployed in order to control their numbers (231). The high number of targets needed to control the vectors of West African sleeping sickness has for a long time made this prohibitively costly. This difference in vector behaviour, in combination with the respective zoonotic and anthropophilic life histories of the parasite, has contributed to the different control strategies for HAT in East and West Africa. The West African disease is controlled mainly through the screening and treatment of the human population whilst vector control plays a bigger role in East Africa.

This hurdle of cost effective West African tsetse control methods has now been overcome after experimentation with target sizes revealed that a 90% reduction in the target area led to a 50% reduction in catch numbers (231). It was found that the number of tsetse caught per m^2 of fabric is optimum with 25 cm^2, targets flanked by an equal area of netting (231). The difference in size between the East African tsetse target and the new West African tiny target can be seen in Fig1.11.

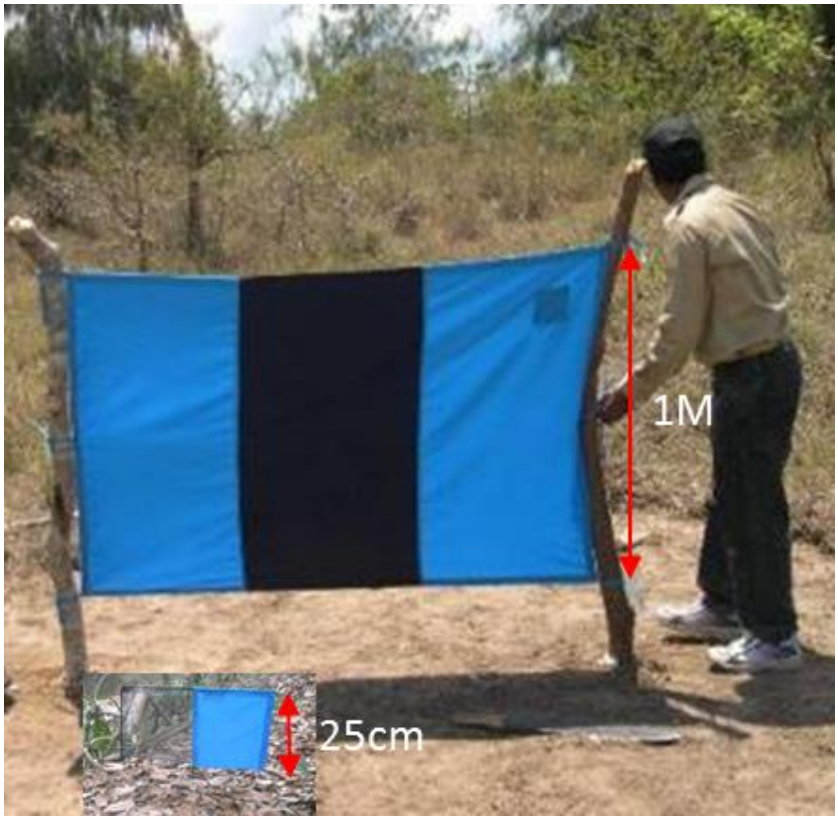


Figure 1.11 ***Comparison of Tiny Target and a larger East African target***
Image used for the tiny target was taken from (232) and the image for the large tsetse target was taken from (233)

Due to their smaller size the reduction in the cost of the fabric offsets the number of targets required for effective control resulting in an economically viable strategy (234, 235). Initial studies, conducted on islands on Lake Victoria, showed that the optimum number of Tiny Targets to be deployed was 20 per linear km (236). The Tiny Targets reduced the tsetse population by >90% and this result was replicated when the Tiny Targets were deployed on a larger scale in NW Uganda in 2013 (236).

1.4.5.5 Insecticide treated cattle

Treatment of cattle with insecticide turns the domestic animals susceptible to nagana into natural bait for the control of tsetse. This control technology was first tested in the Tanganyika territory, in what is today Tanzania, in the 1940s with DDT treated cattle (180). The effectiveness of this method was limited by persistency of the DDT as in order to kill 95% of tsetse that landed on the cattle the DDT needed to be applied twice a week (180). Later trials using the pyrethroid insecticide, deltamethrin, were able to kill 95% of tsetse landing on the cattle without the need for re-application (237). The efficiency costs of treating cattle with deltamethrin can be reduced by 80% with restricted application of insecticide to the belly and legs where >95% of *G. pallidipes* land (182).

1.4.5.6 Insecticidal spraying of resting sites

Just prior to the Second World War in 1939 DDT was discovered to be lethal to insects by the Swiss chemist Paul Müller (238). Following the success of its use by the US Army in WW2 the newly formed WHO established the global malaria eradication program (GMEP) in which adult vector control was to be achieved through application of DDT to the indoor resting sites of malaria mosquitoes (239). The use of DDT, and other organochlorines, to control adult tsetse by spraying trees and bushes followed from the initial success of the GMEP (240, 241). In the 1970s when environmental concerns regarding the toxicity of DDT to the ecosystem were raised (161) DDT was replaced by other synthetic compounds of which the pyrethroids were to play a significant role in tsetse control.

The application of the insecticides to resting places was carried out with the use of pressurised knapsack sprayers. Ground spraying was highly effective but also labour intensive as it required selection and spraying of individual resting sites by an army of technicians. To help cover larger areas mistblowers and unimogs were introduced into some control campaigns (242) (161). Although better than spraying with the knapsacks these methods were still limited by terrain and accessibility issues, limiting the size of the areas that could be controlled.

1.4.5.7 Aerial spraying

Aerial spraying with either residual or non-residual contact ultra-low volume (ULV) insecticides has been used to cover larger areas of terrain inaccessible to the ground based spraying methods. The earliest aerial spraying campaign against tsetse was in 1947 in South Africa (161) using DDT. Pyrethroids were also used in some aerial spraying interventions (243). ULV insecticide spraying involves multiple cycles of insecticide spraying that uses an atomiser to create a fine spray. Multiple cycles are required as the ULV spraying targets the adult tsetse only as the pupating tsetse are safe below ground. The cycles need to coincide with the emergence of new tsetse and were typically spaced 19 days apart. This method of timing meant that the tsetse that had emerged the day after the spraying were killed before they had a chance to deposit their 3rd instar larvae.

1.5 Thesis objectives

Since the early 2000s the number of sleeping sickness cases has been declining in both East and West Africa due to the implementation of successful control strategies. The number of reported West African Gambiense HAT cases has dropped by 89% from 25,865 in 2000 to 2,733 in 2015 (244). Similarly the number of East African Rhodesiense HAT cases has dropped by 90% from 709 in 2000 to 71 in 2015 Fig 1.12 (245).

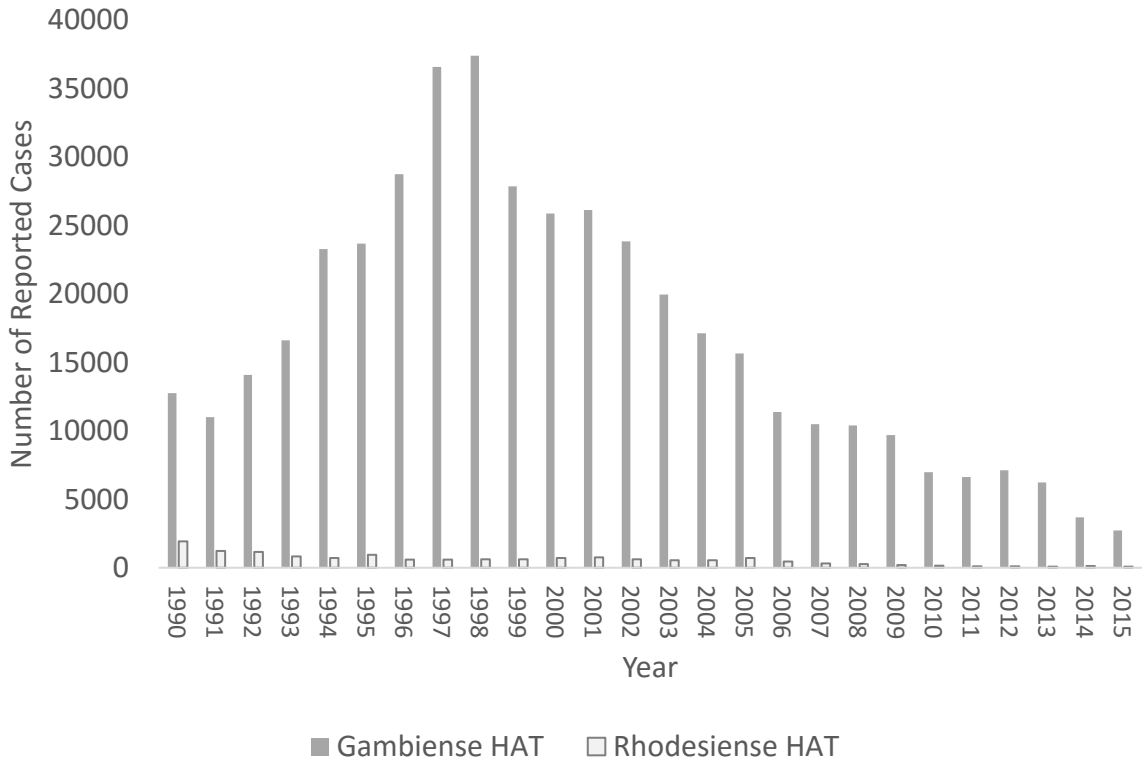


Figure 1.12 ***The number of reported cases of sleeping sickness from 1990 to 2015***
WHO global health observatory data repository

The success of HAT control has resulted in sleeping sickness being included in the London declaration as one of the 10 diseases earmarked for eradication by 2020. To date only two diseases have been eradicated, small pox and rinderpest. This reflects the difficulty of complete disease eradication, this is often due to the difficulty and cost of identifying the remaining transmission sites. As the success of disease control increases it can be harder to justify expending the same cost and efforts to maintain control, especially in resource limited environments. It is therefore important to explore alternative strategies to maintain the pressure on diseases being brought to eradication (246). It is in light of the goals of the London declaration that this thesis will study several aspects of the transmission dynamics

and control of *T. brucei s.l.* in a historic focus of Gambiense sleeping sickness in NW Uganda.

This thesis will examine four research questions that relate to the transmission and control of *T. brucei s.l.* in the historical HAT foci of the West Nile region of Uganda within the context of the elimination goals of the London declaration.

The fours questions addressed are:

1. **What is the prevalence of *T. brucei gambiense* in the tsetse population?**
   This question is to be answered in two parts across two chapters using traditional entomological methods as well as a novel multiplex PCR reaction to assess the prevalence of *T. brucei s.l.* as well as two other salivarian species of trypanosomes, *T. congolense* and *T. vivax*. The study was conducted over 16 months and included physiological, (age) and environmental (rainfall estimates and temperature) factors. The source of bloodmeals was investigated, in order to determine host preference. This data is explored in Chapters 3 and provides a context for the following chapters. The samples positive for *T. brucei s.l.* were then re-screened using sub-species specific primers that target the *T. b. gambiense* specific TgsGP gene, these result are detailed in Chapter 6.

2. **Can commercially available LAMP kits, designed for human screening, be used to screen tsetse for the presence of *T. brucei s.l.* DNA?**
   The previous question answers if *T. brucei s.l.* is present in the local population. This question looks at adapting a pre-existing tool for human surveillance to screen the vector population as an alternative to active screening of people. The current methods used to identify *T. brucei s.l.* in tsetse either rely on classical entomological techniques or molecular methods, such as PCR. Both of these approaches require highly skilled technicians, are time consuming and can be expensive. The recent development of the LAMP technology has the potential to provide the sensitivity and specificity of a molecular assay but without the need for a fully furnished molecular laboratory. This question is fully explored in Chapter 4.

3. **How does the successful deployment of 'Tiny-Targets' effect the prevalence of pathogenic trypanosomes in the local cattle population of the West Nile region of Uganda?**

   The answer to this question is sought by comparing the incidence of three pathogenic trypanosomes, *T. brucei s.l., T. congolense* and *T. vivax* in cattle from two sites in NW Uganda. The first site, Arua, has had Tiny Targets deployed since 2011, the second site, Koboko, has had no tiny target deployment. To identify trypanosome positive cattle both traditional microscopy and standard molecular methods will be used. The analysis of the two cattle populations will also include comparison of relative packed blood cell volumes to identify any relationship between anaemia and infection.

4. **Is *T. b. gambiense* present in the cattle and pigs from the gHAT foci in NW Uganda?**

   There are two sources of sample that are potentially positive for *T. b. gambiense* those samples derived from the vector and those from the vertebrate host. The tsetse samples processed in Chapter 3 are the first source of samples and were screened for *T. b. gambiense*. The second set of samples to screen, vertebrate hosts, are comprised of the cattle sampled in Chapter 5 and pigs from Arua and Moyo. The reason why pigs were selected is that in other studies they have been found to be positive for *T. b. gambiense*, were shown to have a higher prevalence of *T. brucei s.l.* and based on the bloodmeal analysis from Chapter 3 we know that the local tsetse feed on pigs. Currently gHAT is inderstood to be an anthroponosis however there is theoretical and empirical evidence that suggests there may be a role for non-human reservoir hosts in the transmission of the disease. If true, the existence of an animal reservoir host could reduce the chance of reaching the elimination goal set out by the London declaration. The results of screening tsetse for *T. b. gambiense* will be given in Chapter 3 and the results for both the cattle and pig screening assays will be given in Chapter 5.

Chapter 2 : Molecular methods

2.1 Background

This chapter reports the development and/or refinement of molecular methods used in subsequent chapters. These include DNA extraction methods used in Chapters 3 and 5, as well as the different PCR reactions used in Chapters 3, 4 and 5. There are two primary sources of sample that are used in this thesis: tsetse flies and blood samples taken from cattle or pigs that have been collected from the field and stored on Whatman Fitzco/Flinder Technology Agreement (FTA) card. Both the tsetse and blood spot samples were screened for trypanosomes using PCR with either (i) generic, (ii) species-specific and (iii) sequencing to identify the species of *Trypanosoma*.

2.1.1 DNA extraction

FTA cards were chosen as the best means of storing the blood samples of both the cattle and pigs as the technology allows for the safe storage and transportation of DNA at room temperature. The cards are impregnated with four chemicals that lyse cells, denature enzymes, inhibit microorganism growth and protect the DNA from UV damage (247, 248). Previous work has investigated the distribution of trypanosome DNA in blood spotted on FTA cards and found that the DNA is not distributed evenly across the blood spot but rather is only present in specific areas (249). This uneven distribution of trypanosome DNA on the card means that when screening the blood spot the more hole-punches taken increases the likelihood of sampling from an area where trypanosomes DNA is present. The sensitivity of PCRs using processed FTA card samples increases the more hole-punches used, this is especially true for samples with low parasite load. The optimum number of hole-punches taken from a sample depends on whether or not whole blood or lysed blood was applied to the FTA card. With whole blood samples, as used in this study, the sensitivity of the PCR starts to plateau with the processing of 10 hole-punches (250). The use of blood as a sample source is further complicated by the fact that within blood there are inhibitors that can affect the performance of the PCR reaction (251). To reduce the number of inhibitors the hole-punch samples were washed in a solvent. Other inhibitors present in human blood are

heavy metals, which can also be removed with the addition of a chelating agent, such as Chelex, which bind with the heavy metals and remove them from the suspension (248, 252).

2.1.2 Species-specific primers

Since the late 1980s, PCR primers have been developed and used to identify trypanosome species by targeting unique regions of the trypanosome genome that are a signature for the species of interest. Ideally, the target genes would have a high copy number to increase the sensitivity of the primers, as is the case with the Tbr primers, mentioned in Chapter 1. These primers target a 10,000 copy satellite repeat sequence unique to the *T. brucei sl* sub-species group (253) resulting in a highly sensitive and specific PCR assay.

By 1996, a range of species-specific primers had been designed to recognise the most important parasite species which include *T. brucei s.l., T. congolense, T. vivax, T. godfreyi* and *T. simiae* (254). These species-specific primers worked well in furthering our understanding of epidemiology and proved that mixed trypanosome infections in tsetse were more common than previously thought (255-257). However, the design and validation of species-specific primer sets is time consuming and expensive as many screening reactions can deplete the DNA template. A way to reduce the number of reactions, particularly when screening individual samples for different trypanosome species is to run a multiplex PCR. This assay includes more than one primer pair in the PCR reaction allowing several PCR reactions contained within one tube. However, attempts at creating a multiplex primer set for a more varied range of species caused problems due to reductions in sensitivity and specificity (258).

The limit of detection of the species-specific primers varies between species as it is dependent on the genes being targeted by the primers, their copy number and cross reaction with other primer sets. This is especially true for the primers used to differentiate the *T. brucei* sub-species as these reactions rely on the TgsGP and SRA single copy gene regions to distinguish between *T. b. gambiense* and *T. b. rhodesiense*. Other limitations that can arise from using species-specific primers depend on how specific the primers were designed and also the inherent genetic variability of field strains of the different species. A good example of this is the *T. vivax* species complex, which has a large number of different strains with varying degrees of genetic diversity. When species-specific primers were first designed to detect *T. vivax*, the DNA template used to design the primers was subsequently

discovered to be specific for strains from West Africa and consequently the primers performed poorly with East African strains (136). Species-specific primers are designed to detect known species of trypanosomes, not unknown species, and this can lead to an inability to identify microscopy-positive samples (259). This presents a challenge: how do we target unknown trypanosome species when we lack genomic information? Fortunately, we are able to deal with these knowledge gaps by mining known trypanosome genomes for regions that are highly conserved between a wide range of trypanosomes species, which assumes conservation in the unknown species. The resultant primer sets are termed 'generic primers' and their use has helped identify novel trypanosome species in previous surveys (260) and can detect multiple species at the same time reducing time and costs. A good candidate region for generic primer design is the ITS region.

2.1.3 Generic internal transcribed spacer (ITS) primers

The ITS region of the genome is relatively small and highly variable, but it is flanked by conserved regions that are ideal for primer annealing. The ITS variable region differs in size between species and has a relatively high copy number of 100-200 depending on the species. The primers designed in previous studies have been generic so as to successfully amplify known and potentially unknown trypanosome species (144, 145, 147, 261). To increase both specificity and sensitivity, nested PCR assays have been designed that use outer and inner primer sets simultaneously. The product amplified by the outer primers acts as the template for the inner primers (Fig.2.1). If this reaction is performed within the same PCR reaction, it is termed one-step nested PCR, and when using two separate PCR reactions, it is called two-step nested PCR.

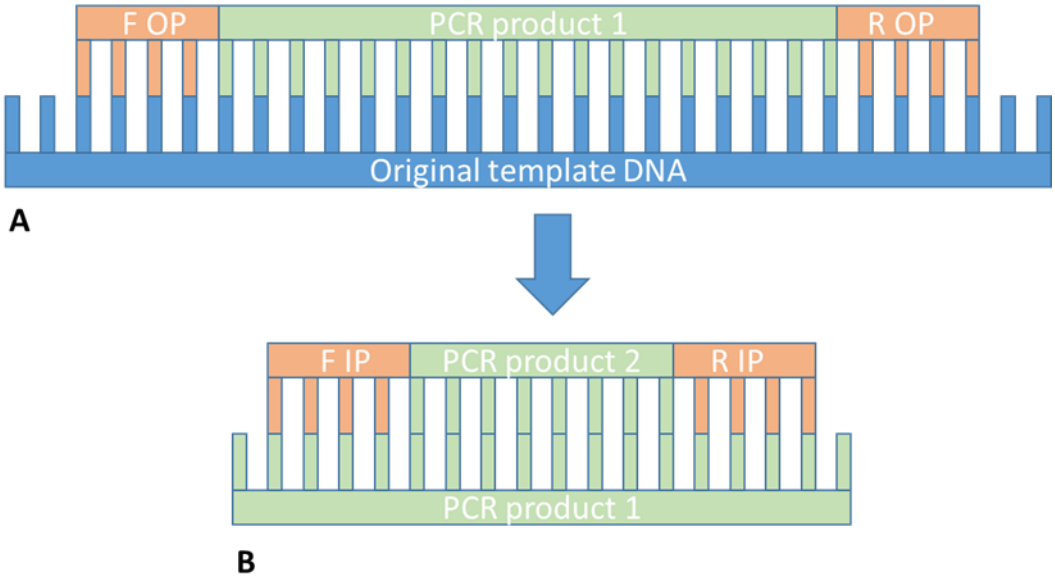


Figure 2.1 ***Diagram of a nested PCR reaction:***
depicting how the outer primers relate to the original DNA template (A) and the inner primers relate to the PCR product generated by the outer primers (B). OP denotes outer-primers, IP inner-primers and F or R refer to the primer position, either forward or reverse.

2.1.4 Sequencing trypanosomes

To check what has been amplified in the PCR reaction and gain further information to differentiate species and sub-species groups, involves a separate technique called DNA sequencing. Primers can be designed specifically for sequencing but it is also possible to have the PCR product sequenced to confirm the predicted identity based on the band size of PCR product.

Two sites commonly targeted for sequence analysis are the 18S ribosomal RNA and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. Both of these genes have a high copy number and because they are essential for eukaryotic cell function they contain, short, highly conserved regions as well as variable regions under evolutionary pressure, resulting in genetic variation. The conserved 18S and GAPDH regions permit the design of generic primers to amplify novel trypanosome species. Using phylogenetic software it is then possible to position the new species in relation to current phylogenetic models.

2.1.5 Limitations of microscopy

The traditional diagnostic microscopy methods used prior to the rise of molecular techniques have been essential for the study of African trypanosomiasis in animals and humans. Microscopy has a very low false positive rate because it requires the identification of live or stained trypanosomes, as opposed to serological and molecular methods, which could be detecting antibodies or DNA from a previous infection which has been cured. Microscopy also has the advantage of being a low cost diagnostic method as it does not necessarily require an electricity source or cold chain, making it ideal for field use, especially in remote rural areas. Microscopy does have several limitations although it can be improved by methods such as the quantitative buffy coat concentration technique (262), however these extra steps increase the resources required and so restrict its use.

The weakness of microscopy is that traditional examination of blood, lymph or cerebrospinal aspirates still have a low sensitivity (263), which can be compounded by low parasitemia in infected individuals. Typically *T. b. gambiense* infections produce fewer parasites than *T. b. rhodesiense* infections, which makes identification of West African sleeping sickness difficult (264). Like many lab-based diagnostic procedures, the skill and knowledge of the technician also greatly influences assay accuracy. This was illustrated in a recent investigation where 50.4% of the laboratories tested failed to identify trypanosomes in a positive blood film (137).

In addition to the problems of sensitivity, microscopy methods cannot morphologically distinguish between sub-species, such as *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense.* Addition of serological methods to microscopic examination can help distinguish some trypanosome infections, such as the development of the card agglutination test for trypanosomiasis (CATT), which is specific for identifying *T. b. gambiense* sero-positive individuals. However, unlike microscopy, this test is limited in that it cannot distinguish between a recently cured person and someone with an active infection (265).

2.1.6 Chapter objectives

The specific aims of the work described in this chapter were to:

1) Evaluate pre-existing molecular techniques for the generic identification of trypanosomes from mammalian blood and tsetse midguts

2) Design new multiplex ITS PCR assays that target a more limited range of trypanosome species compared to the current generic ITS primers in order to better discern between target and non-target species of trypanosome.

3) Quantify the sensitivity and accuracy of the new methods and demonstrate that they are able to three major trypanosome species of interest, *T. brucei s.l., T. congolense* and *T. vivax*.

2.2 Methods

2.2.1 DNA extraction from FTA cards

Blood samples were collected from 2,877 cattle and 766 pigs via a venous ear puncture using a 50mm heparinized capillary tube that holds 35µl of blood. The blood was then spotted onto Whatman FTA cards and processed using a modified version of the method described by Ahmed (250). For each blood spot ten hole-punches (2 mm diameter) were taken using a Harris Micro-Punch™ and placed into a 1.5 ml Eppendorf tube. Between taking each sample, the tip of the Harris Micro-Hole punch underwent three washes, first it was washed in 10% NaClO followed by a wash in distilled water and finally in 100% EtOH, each wash step took five seconds. The tip underwent a final cleaning step by punching 10 holes from clean filter paper to ensure there was no cross contamination between samples and that the tip was dry after being washed. The samples were processed in batches of 72 as this was the maximum number of samples that could be run out on the large gel tanks needed to determine the size of the different trypanosome species when using the generic ITS primers. These 72 samples included three negative hole punch controls, one reagent negative control, one positive hole punch control and one reagent positive control and 66 blood spot samples. The positive hole punch control consisted of 200 µL of *T. b. brucei* (J10) stabilate that had been added to five mL of horse blood, spotted onto FTA cards and left to dry.

Components of blood, such as heme, are known to inhibit the activity of the *Thermus aquaticus* derived DNA polymerase, therefore it was necessary to wash the samples in the following manner. To the hole punches, 1ml of distilled water was added, after sealing the tubes the samples were then rotated using a, Reax 2, overhead shaker (manufactured by Heidolph) for 30 mins at 20 rpm. Following the first wash, two more wash cycles were carried out where the supernatant was decanted and another 1ml of distilled water added, the samples were rotated for a reduced time of 20 mins and 15 mins respectively at 20 rpm. After the final wash the supernatant was removed and the tubes were centrifuged at 10,000 rpm for 15 sec before adding 130µl of a 10% Chelex® (Sigma)-TE suspension and 1.3 µl of proteinase K (20mg/ml, Bioline). Using a Star-Lab Dry Bath System the samples were then heated for one hour at 56°C to allow for proteinase K digestion, which was followed by a

quick vortex. A second heat step at 92°C for 30 minutes to allow for the elution of DNA was done, after which 100µl of the eluted DNA was transferred to a separate tube.

2.2.2 DNA extraction from tsetse tissues

The DNA extraction of the tsetse tissues was modified from the FTA DNA extraction described above. The tsetse tissues were transported in 60µl of 100% ethanol (EtOH) in 96 well PCR plates. To remove the alcohol, the samples were spun down at 10,000 rpm for 30 seconds and the caps removed. The open plates were then placed in a Bio Rad T100^{TM} thermal cycler for two to three hours at 56˚C in order to evaporate the ETOH. Having removed all the alcohol 135 µl of a solution of 1x TE buffer with a 5% Chelex solution and 1.3µl Proteinase K (20mg/mL) was added to each sample. Fresh caps were then placed on the plate and it was once more centrifuged at 10,000 rpm for 30 seconds. The plates were then incubated in a thermal cycler at 56˚C for one hour to allow for the digestion of the tsetse tissues. This was followed by incubation at 93˚C for 30 minutes to elute the DNA and halt the enzymatic reaction.

2.2.3 Blood meal analysis

Having extracted the DNA from tsetse tissues it is possible to screen the samples not only for trypanosomes but also for DNA from blood meals the tsetse fly has recently ingested. To screen for the vertebrate host DNA sequencing primers that target the Cytochrome B (CytB)gene of mammals, reptiles and birds will be used (199), Table 2.1.

The PCR conditions are the same as those described in section 2.2.5 with the exception that 55ºC was used as the annealing temperature.

Table 2-1 ***Vertibrate CytB primers used for the blood meal identification assay***

Primer Name	Primer Sequence 5'-3'	Described by
15_168	TACCATGAGGACAAATATCATTCTG	(199)
15_590	CCTCCTAGTTTGTTAGGGATTGATCG	

2.2.4 Limit of detection (LOD) assay

To ensure that both the ITS and multiplex ITS had comparable sensitivity, both tests were assessed against a 10 fold dilution series of *T. b. brucei* and *T. vivax* DNA. The DNA dilution series (neat to 1/10^7) for each species ranged from 5 ng genomic DNA/uL to 0.0000005

ng/uL. Assuming that the DNA content of each *T. b. brucei* trypanosome is 0.1pg (266) then the dilution series described above ranges between the equivalent of 50,000,000 trypanosomes/mL to 5 trypanosomes/mL.

2.2.5 PCR reaction conditions

The PCR reactions that used Bioline BIOTAQ Red DNA polymerase were carried out at a final volume of 25µL each containing the following reagents: 2.5µl of 10X PCR Buffer (Bioline), 200µM of each of the deoxynucleotide triphosphates (dNTPs) (ThermoFisher), 1.2mM of $MgCl_2$ (Bioline), 0.4µM of both the forward and reverse primers and 10µL of BIOTAQ Red DNA Polymerase (Bioline). The first reaction of the two nested PCRs and standard PCR used 5µL of DNA template. For the nested PCRs second reaction 1µL of the PCR product from the first reaction was used as the template.

For the *T. brucei* s.l. multiplex and *T. b. gambiense* reactions DreamTaq Green was used in a final volume of 25µl consisting of: 12.5µl of DreamTaq Green was used with 0.2µM of each primer and 2µl of DNA template for the *T. brucei* s.l. multiplex; and: 12.5µl of DreamTaq Green with 0.4µM of each primer and 2µl of DNA template for the *T. b. gambiense* specific primers.

The following PCR cycle was used for all three generic ITS primer sets and for the Tbr *Trypanozoon, T. congolense* and *T. vivax* species-specific primers: initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by a final extension step of 72°C for 3 minutes. The PCR reactions were carried out on a BioRad T100™ thermal cycler.

For the *T. brucei* s.l. multiplex reaction and the the *T. b. gambiense* species specific primers the following cycling conditions were used respectively: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 s, 63°C for 90 s, 72°C for 70 s with a final extension step for 10 minutes; and 95°C for 3 minutes followed by 40 cycles of 95°C for 30 s, 63°C for 90 s, 72°C for 70 s with a final extension step for 10 minutes.

2.2.6 Evaluation of current PCR methods

2.2.6.1 ITS PCR evaluation

There are a number of generic primers available that target the trypanosome internal transcriber regions (ITS). Three of these (145-147) were compared with each other to determine their suitability to screen the blood samples collected on FTA cards (Table 2.2). Primer LOD was compared using the dilution series described in section 2.2.3. The primers were also tested with *T. b. brucei* and *T. congolense* serial dilutions applied onto FTA cards. The PCR reactions were carried out as described in section 2.2.5.

Table 2-2 ***Generic ITS primers tested***

Primer Name	Primer Sequence 5'-3'	Described by
Tryp 1R	AAGCCAAGTCATCCATCG	(147)
Tryp 2F	TAGAGGAAGCAAAAG	
Tryp 3F	TGCAATTATTGGTCGCGC	
Tryp 4R	CTTTGCTGCGTTCTT	
ITS1	GATTACGTCCCTGCCATTTG	(146)
ITS2	TTGTTCGCTATCGGTCTTCC	
ITS3	GGAAGCAAAAGTCGTAACAAGG	
ITS4	TGTTTTCTTTTCCTCCGCTG	
CF	CCGGAAGTTCACCGATATTG	(145)
BR	TTGCTGCGTTCTTCAACGAA	

2.2.6.2 Species-specific primers limit of detection

Species-specific primers were used to help confirm the trypanosome species identified using the generic ITS assays. The species-specific primer sets and the target species are listed in Table 2.3.

Table 2-3 ***List of species-specific primers used throughout this thesis***

Primer name	Target Species	Primer Sequence 5'-3'	Expected product size	Reference
Tbr F	*Trypanozoon*	CGAATGAATATTAAACAATGCGCAGT	173	(267)
Tbr R	*Trypanozoon*	AGAACCATTTATTAGCTTTGTTGC		
Tbr-FIND F	*Trypanozoon*	TGCGCAGTTAACGCTATTATACA	117	(268)
Tbr-FIND R	*Trypanozoon*	AAAGAACAGCGTTGCAAACTT		
TCS1	*T. congolense* Savannah	CGAGAACGGGCACTTTGCGA	316	(269)
TCS2	*T. congolense* Savannah	GGACAAACAAATCCCGGGCACA		
TCK1	*T. congolense* Kilifi	GTGCCCAAATTTGAAGTGAT	294	(269)
TCK2	*T. congolense* Kilifi	ACTCAAAATCGTGCACCTCG		
TCF1	*T. congolense* Forest	GGACACGCCAGAAGGTACTT	350	(270)
TCF2	*T. congolense* Forest	GTTCTCGCACCAAATCCAAC		
TVMF	*T. vivax*	TCGCTACCACAGTCGCAATCGTCGTCTCAAGG	399	(51)
TVMR	*T. vivax*	CAGCTCGGCGAAGGCCACTTCGCTGGGGTG		
PLC 657	*T. brucei* s.l.	CTTTGTTGAGGAGCTGCA	324	(271)
PLC 658	*T. brucei* s.l.	CACCGCAAAGTCGTTATT		
SRA 02	*T. b. rhodesiense*	AGCCAAAACCAGTGGGCA	669	(271)
SRA 03	*T. b. rhodesiense*	TAGCGCTGTCCTGTAGACGCT		
VSG 651	*T. brucei* s.l.	GAAGAGCCCGTCAAGAAGGTTTG	>1Kb	(271)
VSG 651	*T. brucei* s.l.	TTTTGAGCCTTCCACAAGCTTGGG		
TgsGP F	*T. b. gambiense*	GCTGCTGTGTTCGGAGAGC	308	(272)
TgsGP R	*T. b. gambiense*	GCCATCGTGCTTGCCGCTC		

With the exception of the PLC, SRA, VSG and TgsGP primers the species-specific primers were tested to determine their LOD using the serial dilution described in section 2.2.3 with the addition of a *T. congolense* Savanna dilution gradient covering the same ng/µL range. The species-specific PCR reactions were carried out using the conditions described in section

2.2.5.

2.2.7 Novel PCR assay development

2.2.7.1 ITS multiplex development

The strength of a generic ITS PCR can also be its weakness as it can be too generic. For example, if it can amplify non-salivarian trypanosome species, this will cause additional complications when trying to identify the species based on banding patterns in an agarose gel. A potential contaminating source of non-tsetse transmitted trypanosomes are the cattle-associated *Trypanosoma theileri* species group (273). The ITS region is variable in size between different strains of *T. theileri.* This results in a range of PCR product band sizes,

approximately 220bp-380bp, which overlap with bands from salivarian trypanosome species, creating false positive identification. Free living flagellates closely related to trypanosomes, such as *Bodo saltans*, can also be amplified.

The ITS-based multiplex primers (mITS) used in the present study were designed to amplify the following three key trypanosome species: *T. vivax, T. congolense* and the *Trypanozoon* species group, which includes both the human and animal parasites. The primers bind to the inner region of the ITS 1 gene between the internal forward and reverse primers described by Adams 2006 (147) as this was the primer set selected to be used in the study.. This design allows for comparable levels of sensitivity between the generic ITS assay and the mITS assay. The outer primers remain the same to retain the sensitivity between the mITS primers and the generic ITS primers and to use the PCR product from the first nest to be useable with both the multiplex and generic primers. The mITS primers were designed by aligning the ITS 1 region from within the inner primers using Mega 6 software (274). The primers were designed to have a universal forward primer and a species-specific reverse primer. The following sequences from the NCBI database were aligned to test forward primer specificity: *T. congolense* Kilifi (TCU22317), *T. congolense* Forest (U22319), *T. congolense* Savannah/IL1180 (U22315), *T. brucei* s.l. (JX910373), *T. vivax* (U22316), *T. godfreyi* (ZWA6307), *T. simiae* (AB742533), *T. theileri* (JX853185) and *Bodo saltans* (AY028452). Reverse primer specificities were tested with the NCBI Primer-Blast tool (275) against other trypanosome species. The primers were also screened against human, cow, pig, tsetse and three tsetse symbionts (*Wolbachia, Wigglesworthia* and *Sodalis*) genomes to test for cross reactivity.

The identification of *T. brucei* s.l., the three *T. congolense* strains and *T. vivax* was based on each species having its own expected band size on amplification with mITS primers. To test potential cross reactivity and specificity, the selected primers were then screened against a library of trypanosome DNA: *T. b. brucei, T. congolense* Savannah, *T. congolense* Forest, *T. congolense* Kilifi, *T. vivax, T. godfreyi, T. simiae* and *T. simiae* Tsavo.

2.2.8 18S and GAPDH sequencing

The sequencing primers targeting the trypanosome GAPDH (276, 277) and 18S (278) genes were tested with the DNA library and four Tryp 1-4 positive cattle blood spots collected

from the field. Despite using DNA samples that had previously yielded reliable results, there were failures in DNA amplification when working with the field samples. Protocol improvements by 1) cleaning the DNA using Qiagen DNeasy Blood and Tissue Kit (279), 2) concentrating the DNA by precipitation, 3) modifying temperature gradients 4) re-ordering and testing primers and 5) testing different taq-polymerases failed to increase primer performance. Consequently, new primers were designed to target the same variable regions as the original 18S and GAPDH primers, but with a smaller product size to ensure that if any DNA degradation was present, particularly in field samples, there would be a better chance of amplification.

These alternative primers were designed by separately aligning the variable regions of both the 18S and GAPDH genes, from a range of trypanosome species, not just the African salivarian species, to help identify the most conserved regions. Where possible the same species were used but there was not a 100% concordance between the species used to design the 18S and GAPDH primers due to what was available on the NCBI online database. To increase their sensitivity, inner and outer sets of primers (nested) were developed. To design the new 18S primers, sequences from *T. b. rhodesiense* (AJ009142), *T. congolense* (AJ009146), *T. godfreyi* (AJ009155), *T. grayi* (KF546526), *T. lewisi* (GU252209), *T. grosi* (AB175623) and *T. otospermophili* (AB175625) were aligned and the primers designed to amplify the 500-600 bp variable region. To design the GAPDH primers the following sequences were aligned, *T. b. rhodesiense* (AJ620284), *T. congolense* (AJ620291), *T. simiae* Tsavo (FM879137), *T. vivax* (AF053744), *T. grayi* (FM164795), *T. lewisi* (AJ620272) and *T. grosi* (AB362557). The primers were then tested using the NCBI Primer design tool (275) to check for kinetoplastid specificity. The new primers were again screened against human, cattle, pig and tsetse genomes, including the bacterial tsetse symbionts.

2.3 Results

2.3.1 Evaluation of current PCR methods

2.3.1.1 ITS PCR evaluation

Three primer sets were tested with the trypanosome DNA library kindly supplied by Professor Wendy Gibson from Bristol University. All three primer sets performed well and produced PCR products with bands of the expected size (Table 2.4).

Table 2-4 ***The band sizes (bp) produced by each of the ITS trypanosome primer sets.***

Primer set	Trypanosome species	Final product size (bp)
Tryp 1-4 (nested)	T. congolense Savannah	640
	T. congolense Forest	640
	T. congolense Kilifi	560
	Trypanozoon	430
	T. simiae	370
	T. simiae Tsavo	350
	T. grayi	350
	T. godreyi	240
	T. vivax	180-200
ITS 1-4 (nested)	T. congolense Savannah	1413
	T. congolense Forest	1513
	T. congolense Kilifi	1422
	Trypanozoon	1207-1224
	T. simiae	850
	T. simiae Tsavo	980
	T. grayi	940
	T. godreyi	650
	T. vivax	611
ITS CF-BR	T. congolense Savannah	700
	T. congolense Forest	700
	T. congolense Kilifi	620
	Trypanozoon	480
	T. simiae	396
	T. simiae Tsavo	370
	T. grayi	370
	T. godreyi	300
	T. vivax	240

The sensitivity of the different Generic PCRs was tested with a 10 fold dilution series of *T. b. brucei* and *T. vivax* DNA. The two nested primer sets (Tryp 1-4 and ITS 1-4) had a higher sensitivity than the simple PCR primers (CF-BR); the latter started to lose sensitivity after 0.5

ng/µL and only produced a very faint band with 0.05 ng/µL of template, whereas both nested PCRs produced strong bands at this concentration and only failed to amplify the target DNA after 0.005ng/µL

Once these primers were validated, they were tested on DNA extracted from blood spots preserved on FTA cards where varying concentrations of trypanosomes had been added (1 x 10^2 trypanosomes/mL to 1 x 10^6 trypanosomes/mL). The simple ITS CF-BR PCR assay again had reduced sensitivity and was unable to identify the lowest concentration of *T. congolense* trypanosomes. The nested primer set, ITS 1-4 also failed to amplify the lowest concentration of the *T. b. brucei* and *T. congolense* sample. Only the Tryp 1-4 (nested) primers correctly identified all the trypanosome-spiked samples, including the lower concentrations of *T. congolense* and *T. b. brucei.* Based on these results, the generic ITS 1 primers described by Adams et al 2006 would be used to screen all future blood samples (Fig2.2).

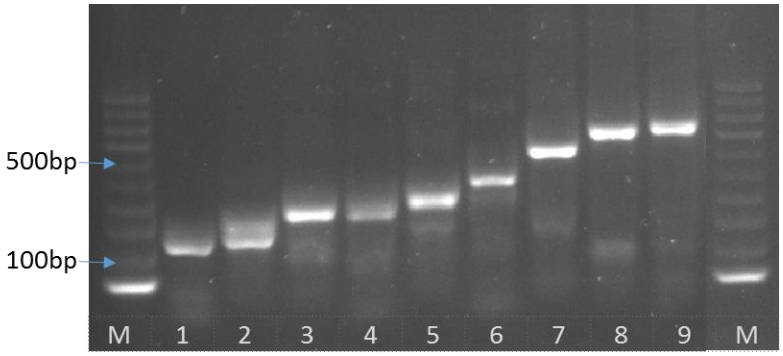


Figure 2.2 ***Gel image of the relative sizes of the salivarian trypanosomes:***
(1) *T. vivax*, (2) *T. godfreyi*, (3) *T. grayi*, (4) *T. simiae*, (5) *T. simiae* Tsavo, (6) *T. brucei s.l.*, (7) *T. congolense* Kilifi, (8) *T. congolense* Savannah, (9) *T. congolense* Forest and (M) marker

2.3.1.2 Species-specific primers

Compared to the Tryp 1-4 and mITS nested primers, the limit of detection for the species-specific primers varied. The species-specific primers for *T. congolense* and *T. vivax* were less sensitive than either of the nested primers failing to detect any DNA beyond 0.05ng/µL, however both the *T. brucei s.l.* specific primers (Tbr and Tbr-FIND) were more sensitive than

the nested primers with the ability to amplify 0.000005ng of DNA/µL, the equivalent of 50 trypanosomes/mL.

2.3.2 Novel PCR assay development

2.3.2.1 Multiplex ITS primers (mITS)

Having aligned the sequences for *T. congolense* Kilifi (TCU22317), *T. congolense* Forest (U22319), *T. congolense* Savannah/IL1180 (U22315), *T. brucei* s.l. (JX910373), *T. vivax* (U22316), *T. godfreyi* (ZWA6307) and *T. simiae* (AB742533), the primers in Table 2.5 and Fig 2.3, were designed and tested using the NCBI Primer-basic local alignment search tool (BLAST) (275) against the Genome database (from all organisms).

Table 2-5 ***Novel multiplex ITS 1 sequences and PCR product sizes***

Primer name	Sequence 5'-3'	Target organism	Product size	Tm˚C
MpMk2F	TAGCTGTAGGTGAACCTGCAGC	*Trypanosomatidae*	-	63
MpMkTcR	GCGTCAGGCGGCRWAAGAA	*T. congolense*	392, 433*	63
MpMkTbR	ATGCGAGGTTGATATACACATAGCA	*T .brucei sl*	342	63
MpMkTvR	GCCGTGCTCCACCTG	*T .vivax*	139	63

*two different sizes of product due to *T. congolense* Kilifi having a shorter ITS 1 region than *T. congolense* Savannah and *T. congolense* Forest.

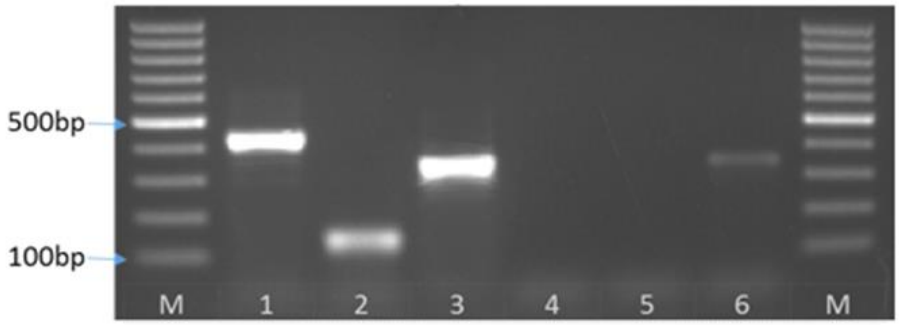


Figure 2.3 ***Gel image showing the relative sizes of the mITS PCR reaction:***
(1) *T. congolense* Savannah, (2) *T. vivax* and (3) *T. b. brucei*. Lanes 4-6 comprised of two negative reagent controls and a single *T. b. gambiense* positive control respectively. The nucleotide weight marker (M) used was the 100bp ladder from Invitrogen

The following *in silico* conditions were used to design the primers: product size = 100 bp +/- the target product size, primer melting temperatures (Tm) 61˚C - 65˚C with a max Tm difference of 2˚C. The remaining test conditions were left as the default settings.
There was some cross priming detected with other species, most notably with the *T. vivax* reverse primer, however these products were beyond the size range specified, (max 100 bp

+/-) of the target product size. Dilution assays of *T. b. brucei* and *T. vivax* demonstrated that both the original generic ITS 1 primers designed by Adams (2006) and the new multiplex primers have a similar detection range with a lower limit of 0.05 ng/uL for the trypanosome species tested.

2.3.2.2 18S and GAPDH

The new 18S and GAPDH primers are listed in Table 2.6. They were tested against the trypanosome DNA library and produced the expected sized products. These were sent for sequencing and BLASTed to confirm identity. Both sets of primers were able to amplify the 18S and GAPDH regions correctly for each trypanosome species they were tested against. When tested against the specified non-target organisms, no products with 100 bp +/- pf the expected product size were produced.

Table 2-6 *Novel sequencing primers for* Trypanosoma

Primer name	Sequence 5'-3'	Target organism	Product size*	Tm˚C
GAPDHFout	TACTTYGCVTAYCAGATGMRSTACG	*Trypanosoma*	789	55
GAPDHRout	GCTKCGRYTGTCRYTGATGA	*Trypanosoma*	789	55
GAPDHFin	CACCAYGTBGTGTCSAACG	*Trypanosoma*	349	55
GAPDHRin	CRATCTCCTTGATGCBTGTGT	*Trypanosoma*	349	55
18S Fout	TAATTCCAGCTCCAAAAGCG	*Trypanosoma*	713-814	55
18S Rout	CGTGCTGAGGAYATTCCCGT	*Trypanosoma*	713-814	55
18S Fin	TCGTAGTTGAAYTGTGGGC	*Trypanosoma*	515-599	55
18S Rin	GCAGTGTGGACTACAAT	*Trypanosoma*	515-599	55

*there is a size variation amongst the products produced by the 18S primers depending on the species of trypanosome being amplified.

Both sets of sequencing primers were also tested with a serial dilution of *T. b. brucei* and *T. vivax* DNA. The GAPDH primers had a higher sensitivity than the ITS 1 and mITS primers when tested against the *T. brucei* DNA with a lower limit of 0.005 ng/µL and a comparable sensitivity when tested against the *T. vivax* DNA. However the 18S primer was more sensitive than the ITS 1 and mITS primers by a factor of two when tested against the *T. b. brucei* concentration gradient and by a factor of one against the *T. vivax* assay with lower limits of 0.0005 ng/µL and 0.005 ng/µL, respectively, Fig 2.4. Due to the difference in sensitivity, only the 18S sequencing primers were taken forward to be used in the rest of the thesis.

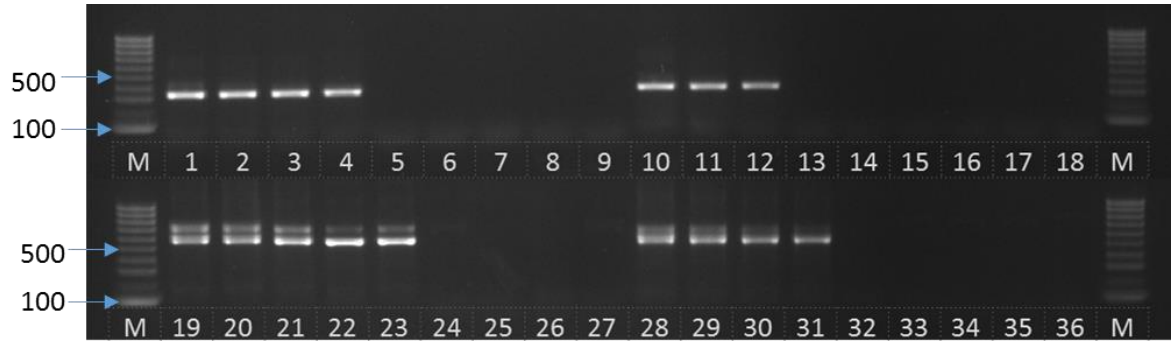


Figure 2.4 ***Gel image of the GAPDH (1-18) and 18S (19-36) limit of detection assay:***
(1-8, 19-26) and *T. vivax* (10-17, 28-35). Negative controls were included (9, 18, 27 and 36) plus molecular weight markers (M).

2.4 Discussion

2.4.1 Generic ITS primer set

Of the three generic ITS-based primer sets screened, the primers named Tryp 1-4 proved most promising due to their higher sensitivity and absence of non-specific DNA amplification. The lower detection limit of Tryp 1-4 was 0.05 ng/µl of trypanosome DNA based on dilution series of *T. b. brucei* and *T. vivax*.

2.4.2 Multiplex ITS (mITS) primers

A set of multiplex primers was designed to amplify the specific subset of trypanosome species of medical and veterinary importance. This was done using a universal forward primer and species-specific reverse primers for the *Trypanozoon* group, *T. congolense* and *T. vivax*. The mITS primers were designed for use with the Tryp 1-4 ITS outer primers to ensure that the sensitivities between the two primer sets were the same, thus permitting comparison between the two sets. The outer Tryp 1-4 primers can be used with the original inner primers and the mITS primers as both have equivalent sensitivity. When tested against non-specific gene targets, the mITS did not amplify non-target salivarian trypanosomes, *T. theileri* and *B. saltans*, which confirms that the presence of these non-target organisms in samples will not confound the multiplex assay.

2.4.3 Sequencing primers for 18S and GAPDH

Primers designed to amplify the 18S and GAPDH gene sequencing regions successfully amplified all nine trypanosomes in the DNA library. When tested against field isolates, the primers amplified DNA products of the correct size and of suitable quality for sequencing.

The sensitivity of these sequencing primers are equivalent to the nested ITS primers used to initially identify samples of interest. Sequencing primers can be used to confirm the identity of amplified samples by quickly sending products off for sequencing without delay. GAPDH and 18S primer sets also help confirm sample identities made with Tryp 1-4 and mITS primers. Furthermore, when unknown trypanosome species are identified, these primers are crucial for getting sequence data that can place new species in the correct phylogenetic position relative to currently known trypanosomatids.

2.4.4 Species-specific primers

Species-specific primers can identify trypanosomes without the added complication and expense of sending PCR products for DNA sequencing. However, of the eight species-specific primer sets selected for parasite identification, only the TBR primers (that target the *T. brucei sl* sub-species) had a greater sensitivity than the nested ITS primers. This difference in sensitivity is due to the nested nature of the generic ITS primers and a difference in copy number of the genomic sequence that the ITS and Tbr primers target. Therefore, the Tryp 1-4 and mITS primers, because of the high copy number of target gene, are more likely to detect lower amounts of trypanosome DNA than the species-specific primers, with the exception of the Tbr primers. Additionally, the generic nature of Tryp 1-4 primers enhances the amplification of local strains that may not react with the more precise species-specific primers. However, using species-specific primers offer a more convenient and affordable means to quickly confirm most positive samples identified by generic PCR assays than sequencing.

2.4.5 Final primer selection

Having assessed the primers described in this chapter the following primer selection was made, Table 2.7, to be used throughout the rest of the thesis.

Table 2.7 Final selection of primers to be used throughout the rest of the thesis for the identification and detection of trypanosomes

Primer set	Target species	Final product size (bp)	LOD ng/µl
Tryp 1-4 (nested)	*T. congolense* Savannah	640	0.05
	T. congolense Forest	640	
	T. congolense Kilifi	560	
	Trypanozoon	430	
	T. simiae	370	
	T. simiae Tsavo	350	
	T. grayi	350	
	T. godreyi	240	
	T. vivax	180-200	
mITS (nested)	*T. congolense Savannah*	433	0.05
	T. congolense Forest	433	
	T. congolense Kilifi	392	
	Trypanozoon	342	
	T. vivax	139	
18S (nested)	*Trypanosoma*	349	0.005-0.0005*
Tbr-FIND	*Trypanozoon*	117	0.000005
TCS	*T. congolense Savannah*	316	0.5
TV	*T. vivax*	399	0.5
PLC	*T. brucei s.l.*	324	
SRA	*T. b. rhodesiense*	669	
VSG	*T. brucei s.l.*	>1Kb	
TgsGP	*T. b. gambiense*	308	

*These values are only applicable to *T. vivax* and *T. brucei s.l.*, LOD for *T. congolense* was not determined.

Chapter 3 : Prevalence of *T. brucei s.l.* and other *Trypanosoma* spp. in *G. f. fuscipes*

3.1 Background

3.1.1 Tsetse as vectors of HAT and AAT

The tsetse fly, genus *Glossinidae*, is the sole vector for the two trypanosome sub-species responsible for human African trypanosomiasis. They are also the most important vectors of Trypanosoma spp. which cause animal African trypanosomiasis, albeit horseflies (Tabanidae) are able to act as mechanical vectors for veterinary important species of trypanosomes (280, 281). Due to their ability to act as mechanical vectors, tabanids have been able to spread *T. vivax* outside of Africa amongst South American cattle populations (282).

The study of the vector population is important in understanding the transmission of disease as key aspects of vector biology have been shown to affect disease transmission, these include the species of local tsetse present (120, 283-285), the age structure of the tsetse population (203, 286-288), sex (289) and host preference (290, 291) .
There are 30 species of tsetse and these are assigned to one of three species groups, *Mositans* (Glossina)*, Palpalis* (Nemorhina) and *Fusca* (Austeni) Table 3.1. Of these 30 species only six are responsible for the majority of sleeping sickness cases, *Glossina palpalis, Glossina fuscipes, Glossina tachninoides, Glossina morsitans, Glossina pallidipes* and *Glossina swynnertoni* (156, 292). These six species all belong to only two of the species groups *Palpalis* and *Morsitans*. The third group of tsetse flies, *Fusca* is not considered a vector of HAT as it rarely feeds on humans and typically occupies habitat that is removed from urban and agricultural lands. The species of tsetse responsible for the spread of nagana include the five associated with HAT as well as *Glossina austeni* and *Glossina longipalpis* and the following members of the *Fusca* group: *Glossina brevipalpis, Glossina fusca, Glossina tabaniformis* and *Glossina vanhoofi*.
The susceptibility of tsetse to infection with *T. brucei* is primarily dependent on the age of the tsetse, newly emerged flies, termed teneral flies, are more susceptible to infection from trypanosomes compared to older flies although starvation can also render older flies

susceptible to infection (293). Other factors that can contribute to a mature trypanosome infection establishing in tsetse are the vector species (120) , the sex of the tsetse (294), temperature (295), the specific strains of trypanosome parasites (296, 297) and even the microbiome of the tsetse with special significance placed on the presence of the symbiont bacteria *Sodalis glossinidius* (298).

Table 3-1 ***A list of all 30 species of tsetse detailing their respective habitats***

Group	*Glossina* species	Habitat
Morsitans	*G. longipalpis* *G. pallidipes* *G. morsitans morsitans* *G. morsitans submorsitans* *G. morsitans centralis* *G. swynnertoni* *G. austeni*	Woodland savannah
Palpalis	*G. palpalis palpalis* *G. palpalis gambiensis* *G. fuscipes fuscipes* *G. fuscipes martinii* *G. fuscipes quanzensis* *G. tachinoides* *G. pallicera pallicera* *G. pallicera newsteadi* *G. caliginea*	Riverine and swamp vegetation
Fusca	*G. nigrofusca nigrofusca* *G. nigrofusca hopkinsi* *G. fusca fusca* *G. fusca congolensis* *G. fuscipleuris* *G. haningtoni* *G. schwetzi* *G. tabaniformis* *G. nashi* *G. vanhoofi* *G. medicorum* *G. severini* *G. brevipalpis* *G. longipennis**	Rain Forest and Riverine Forest

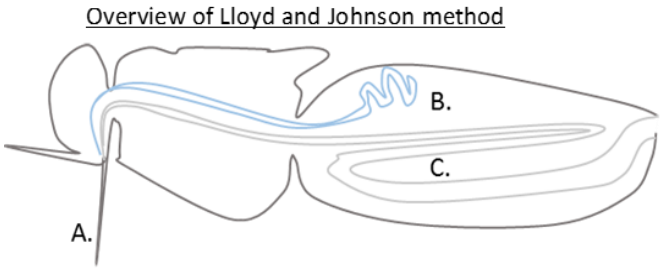
**G. longipennis* differs from the other Fusca species in that it is found in savannah habitats and not in forest habitats.

Salivarian trypanosomes take varying amounts of time to establish a mature infection in their respective tsetse hosts, this is temperature dependent with *T. vivax* taking between five days at 29°C to 13 days at 22°C (295). *T. brucei s.l.* has been shown to take between 17 to 45 days (299) to establish a mature infection with a study on *T. b. rhodesiense* showing that at temperatures of 28°C and 30°C it took 23 and 12 days respectively to establish salivary gland infections (300). Studies on *T. congolense* have shown at 23°C to 24°C it takes

15 to 20 days for a mature infection to establish (285) although other studies have shown that it can take 19 to 53 days for *T. congolense* to establish an infection (299). The sex of the tsetse is another aspect of the biology that has been shown to affect the ability of trypanosomes to establish an infection. Male tsetse are more susceptible to the establishment of mature salivary gland infections in the species *G. m. morsitans* and a similar difference between the sexes was seen in *G. pallidipes* however it was less pronounced (294).

3.1.2 Identification of infection

The Lloyd and Johnson method of assessing infection of tsetse with trypanosomes (135) is a traditional technique for identifying three of the major trypanosome sub-genera based on the tissue type that trypanosomes are found in after dissection (Fig 3.1). The identification of trypanosomes in either the salivary glands, midgut or mouthparts of the tsetse are used, in theory, determine whether the trypanosomes are either *Trypanozoon, Nannomonas* or *Duttonella*. The Lloyd and Johnson technique which was developed to identify the species of trypanosome in the tsetse has limitations (259) as it cannot identify mixed infections or distinguish between species that invade the same organs, e.g., infections of T. brucei and T. congelense in the midgut. However, the traditional dissection technique can inform on whether the fly has a mature infection and the location of the parasites can provide a preliminary overview of what to expect in later, more sensitive, molecular assays.



A. mouthparts, B. Salivary glands, C. midgut

Sub-genus	Salivarian species	Location of trypanosomes immature infection	mature infection
Nanomonas	*T. congolense*	midgut	mouthparts
Trypanozoon	*T. brucei*	midgut	salivary glands
Duttonella	*T. vivax*	mouthparts	mouthparts

Figure 3.1 ***Trypanosome development in the tsetse***
Image adapted from FAO handbook for tsetse control (159). Using this method trypanosomes found in the mid gut would be classed as either *T. congolense* or *T. brucei* s.l., those found in the mouthparts would either be *T. vivax* or *T. congolense* whilst those found in the salivary glands would be indentified as *T. brucei* s.l..

As previously mentioned (Chapter 1) the use of molecular methods for identifying trypanosomes greatly increases the resolution of prevalence of different trypanosome species with regards to the sensitivity and specificity.

3.1.3 Ageing tsetse

As well as identifying infected flies, classical dissection methods also allow the age of flies to be estimated either through the wing fray method (301) or through ovarian dissection (302). The importance of understanding the ages of the vector population allow for assessment of transmission risk, with older flies more likely to have a mature infection (303, 304). The two ageing methods that can be used vary in their application and accuracy, the wing fray method can be applied to both male and female tsetse but is considered less accurate as it is dependent on the activity of the individual fly, which may vary not just between individuals but between seasons and the sampling method (305).

The ovarian method used to determine the physiological age of tsetse was adapted by Saunders (302) from the methods developed by Soviet scientists for determining the age of

mosquitoes (306). To carry out this method the ovaries and uterus of the tsetse are removed, once removed it is possible to observe the different developmental states of the four oocytes found in the ovaries. These four oocytes are termed the left inner, right inner, left outer and right outer due to their relative position within the female, with the inner oocytes located more anteriorly than those termed outer oocytes (Fig 3.2).

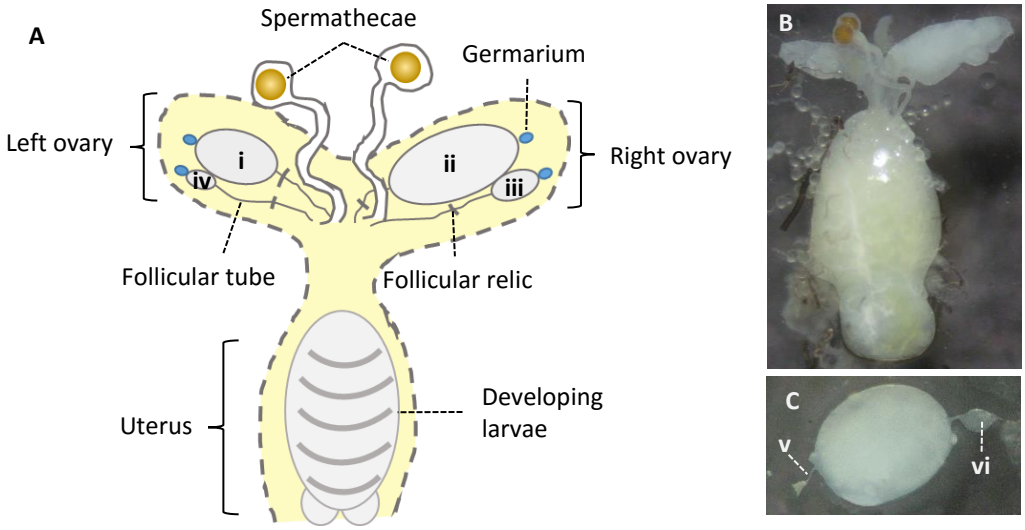


Figure 3.2 Female tsetse reproductive system:
(A) diagram of the female tsetse reproductive organs, with the four oocytes labelled as follows: (i) Right inner oocyte, (ii) Left inner oocyte, (iii) Right outer oocyte, (iv) Left outer oocyte**. (**B**)** an example of an ovarian dissection showing the correct orientation with the spermathecae overlaid on top of the ovaries. The developing larvae can be clearly seen in the uterus. (C) the right outer oocyte has been removed from the right ovarie and here we are able to see the follicular tube with a relic attached (v) and the germarium (vi). Image adapted from the FAO training handbook (159)

This method takes advantage of the sequential development of the oocytes and the known time it takes for the completion of a gonotrophic cycle (Fig 3.3). This method can only be used with fertilised females and any delay in fertilisation would skew the physiological age of the tsetse from the chronological age. The extent of this skewing is slight as the probability of finding un-inseminated females a week after emergence is very low (307). Research conducted with *G. pallidipes* and *G. m. morsitans* found the probability of finding a virgin female seven days after emergence was 10% and 0% respectively (308).

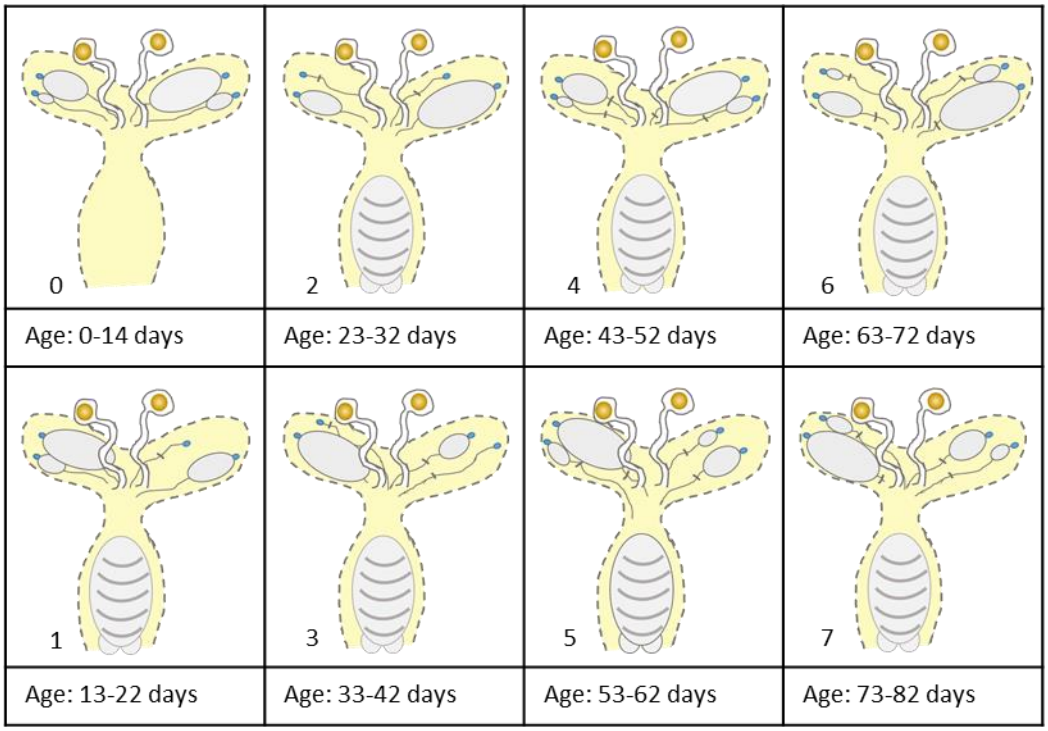


Figure 3.3 ***Ovarian categories (0-7) and their age estimates***
Image adapted from the FAO training handbook (159) showing the relative development of the ovaries and their corresponding age estimation.

3.1.4 Host blood meal identification

Blood meal analysis in the study of disease transmission allows the researcher to identify the main hosts of the hematophagous insect vectors. The ability to identify what animals the tsetse are feeding on provides information on the range and relative importance of hosts. Monitoring tsetse feeding in the field and observing them feeding on host animals can be problematic as the presence of a field worker trying to observe this behaviour may in itself attract or repel flies depending on the species (187, 309). An alternative to observing the active feeding of tsetse on animals is to analyse their gut contents in order to identify which animal species they have fed upon. The basis of analysing the gut contents of hematophagous insects relies on the identification of markers that are specific to the hosts. These markers must be stable so as not to degrade to quickly over time and they must occur in relatively high amounts so that they can be detected in the low volume of an insect blood meal. The two most commonly used types of markers for blood meal identification are proteins and DNA.

The detection and identification of different host specific proteins relies on the antigenic relationship of different animal sera with their phylogenetic classification. This principle was

first demonstrated in 1904 by Nuttall (310) with his experiments into blood precipitation tests. In these experiments, he was able to demonstrate that, when mixed, the serum of two different species formed a precipitate and that this reaction was more distinct the less related the two species were. This method was adapted for use in the investigation of arthropod blood meals (311, 312). The precipitin test is not commonly used as it lacks specificity due to cross-reaction between phylogenetically close species and it is expensive as it requires a large volume of antibodies (313).

Due to the poor sensitivity of the precipitin test a more sensitive serological test was developed by Weitz (314) known as an inhibition test. Despite being more sensitive than the precipitin test (315), it still resulted in cross reactions and was technically more complex making it ill-suited for field work. In 1986, the enzyme-linked immunosorbent assay (ELISA) was developed for the identification of blood meals in haematophagous insects by Service (316). The ELISA test uses immobilised antibodies that had been raised against specific host species. The ELISA tests were less complex and could be used in a kit form making them more suitable for field work. A study by Clausen in 1998 using the ELISA technology was used to screen 29, 245 wild caught tsetse blood meals. In total, 62.8% of the blood meals were identified down to either host-group, (e.g. ruminant), family or species (172). These antigenic methods require the screening of the blood samples against pre-prepared host immune sera, therefore the more potential hosts there are the larger the number of immune sera that need to be prepared, making these methods labour intensive. These serological methods have a low specificity when distinguishing between closely related species such as species of antelope (172).

The use of molecular methods to identify vector blood meals has been used since the early 1990s and has the advantage of being able to not just distinguish between different host species but is also able to use genetic markers to discriminate between individuals within a village (317) or group of animal hosts (318) The other advantage of molecular methods is that it is possible to amplify the DNA allowing for greater analysis from a smaller starting volume, making the process highly sensitive(319). As discussed previously (Chapter 2, section 2.2.3), the cytochrome b region of the genome can be used to identify the source of blood meals. This gene is conserved across genera and is often used as a target to identify

the origins of blood meals in haematophagous arthropods (198, 320, 321). There have been various assays developed targeting this region, designed to be read on a gel or submitted for sequencing (322, 323).

3.1.5 Objectives

North Western Uganda is a historic hot spot for gambiense sleeping sickness (56). Using both classical entomological methods and molecular methods this chapter describes studies conducted to assess the prevalence of *T. brucei s.l., T. congolense* and *T. vivax* in the tsetse population. As mentioned in section 3.1.1 the prevalence of trypanosome infection in tsetse populations is dependent on physiological and environmental factors, which this chapter will assess. The overall aim of the work described in this Chapter was to assess the prevalence of *Trypanosoma* spp. in the tsetse population of the Koboko focus in NW Uganda. To achieve this aim, the the specific objectives were:

1. Using traditional and molecular methods, assess the prevalence of *T. brucei s.l., T. congolense* and *T. vivax* in the local tsetse population
2. Quantify variation in the age structure, sex ratio and relative abundance of tsetse across a period of 16 months from April 2013 to July 2014.
3. Assess the impact of temperature and rainfall on the abundance, age structure and sex ratio of the tsetse population
4. Using the molecular methods to screen for vertebrate cytochrome b DNA estimate the diet of the tsetse population.
5. Screen the Tbr positive tsetse samples for *T. b. gambiense* using the *T. brucei* s.l. multiplex primer assay and the TgsGP primers.

3.2 Methods

3.1.5 Study site

The study site was located along the Kochi River in the Koboko district of NW Uganda. The Kochi is a perennial river that flows through Koboko allowing for tsetse sampling throughout the year. Traps were deployed between 3.451°-3.465° N and 30°58'-31°03'E. This is an agricultural area where crops such as cassava, tobacco, millet and sesame are grown. The human population of Koboko was 204,048 in 2014 (324). The most recent census on the livestock numbers raised in Koboko (325) are given in Table 3.2. Of the mammalian livestock animals, the low number of pigs is a reflection of Islam being the predominant religion in the area.

Table 3-2 ***Census numbers from 2008 of the different domestic animals present in Koboko***

Livestock	Number	Percentage of total livestock population
Cattle	54204	13.2
Goat	101602	24.8
Sheep	33250	8.1
Pig	275	0.1
Chicken	209513	51.2
Ducks	9742	2.4
Turkeys	648	0.2
Total livestock population	409234	

The NW of Uganda is a historic focus for *Gambiense* sleeping sickness cases, with the disease being reported from 1905 (326) to the present day. The number of gambiense-HAT (gHAT) cases reported from the area has decreased from 948 in 2000 to only four cases in the first 10 months of 2016 (327). This makes the NW of Uganda a good example of a gHAT focus close to elimination. The main vector of *T. b. gambiense* in NW of Uganda is *G. f. fuscipes* (159, 236, 328), a member of the palpalis group and one of the six species of tsetse responsible for the majority of sleeping sickness transmission.

3.2.1 Tsetse catches

A total of 6,664tsetse flies were caught along the Kochi River in the district of Koboko over 16 months from April 2013 to July 2014, Fig 3.4. These flies were caught at a total of four different trap sites (KO16, KO19, KO20 and KO21) using standard pyramidal traps (329) Fig 3.5.

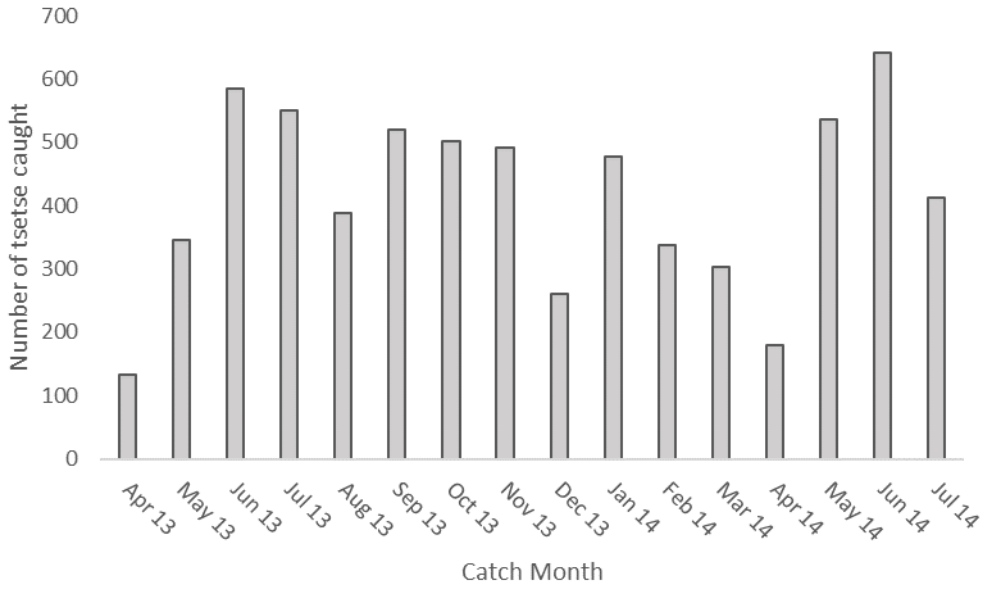


Figure 3.4 Tsetse catch numbers from the Kochi river from April 2013 to July 2014

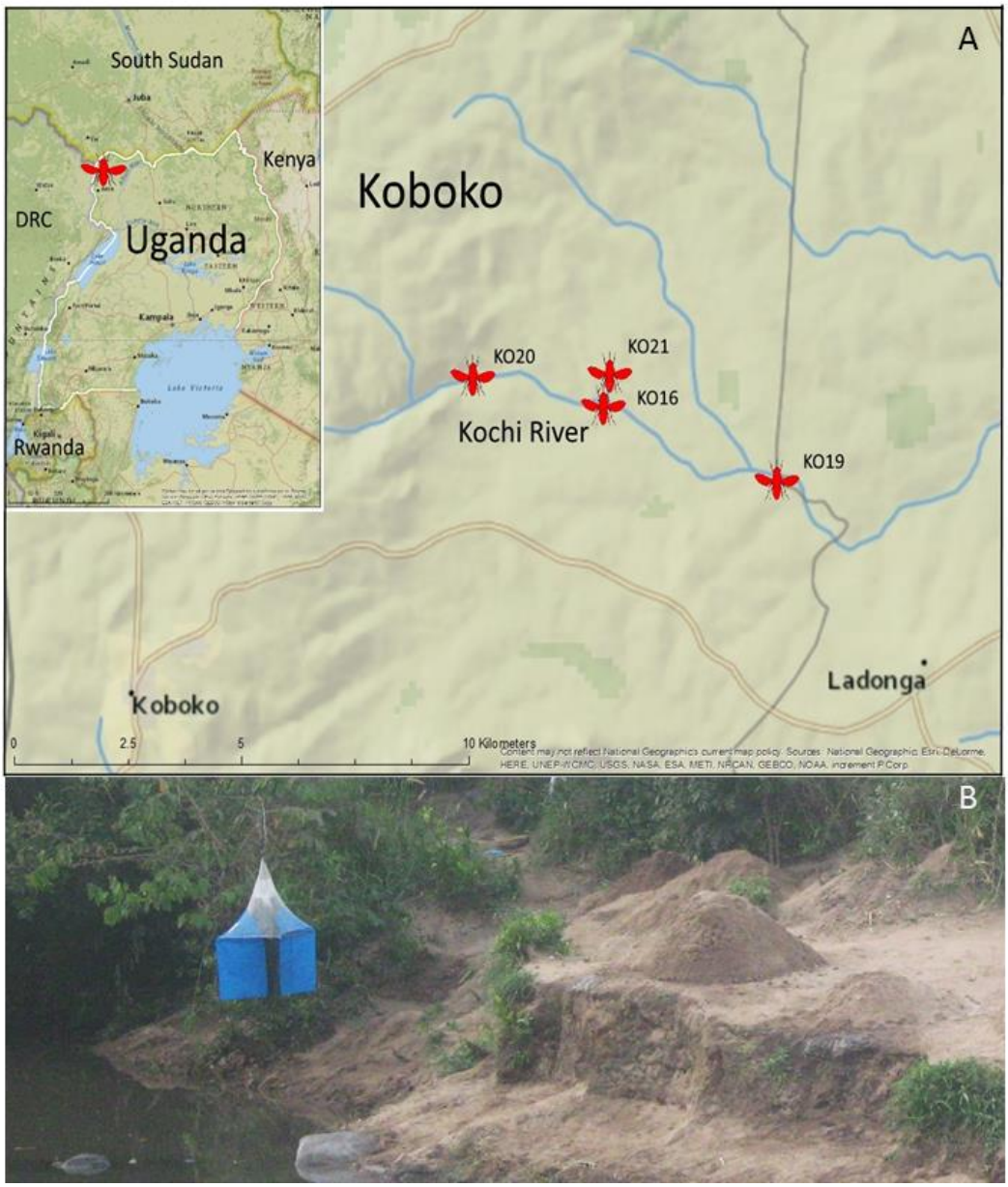


Figure 3.5 A map of the tsetse study site:
Trap sites used are denoted by a red tsetse symbol, *B.* ***pyramidal trap set up along the Kochi River.***

The traps were deployed <2m from the river and grease was smeared on the branch supporting the trap to prevent ants getting into the trap and carrying off any caught flies. The traps were checked twice a day, once in the morning at 7:30 h and again in the afternoon at 15:30 h, traps with tsetse were removed and replaced with a fresh trap. The tsetse-laden traps were transported back to the field laboratory in chilled cooler boxes with a damp cloth to reduce heat stress and mortality.

3.2.2 Dissection

On arrival to the laboratory, the traps were emptied and the live and dead tsetse were sorted based on eye colour and we also took this opportunity to sex the flies based on their external genitalia. The tsetse were dissected by trained laboratory technicians using a Zeiss Stemi 2000 dissection microscope equipped with the C LED stand. The three tissues of interest, mouthparts, salivary glands and midgut, were then screened using one of two compound microscopes, either a Zeiss Primo Star or a Leica DM500. Both these microscopes were fitted with a dark-field filter to increase contrast when screening samples for living trypanosomes.

The dissection first required the removal of the head and salivary glands by carefully pulling the head off from the thorax, the salivary glands still being attached to the head are then pulled out of the body. The head and salivary glands were then transferred to a separate drop of saline on the same slide and here the salivary glands are separated from the head. Having removed the salivary glands, the body of female flies was further dissected by making two small incisions either side of the second abdominal segment after which the ovaries, spermathecae and uterus were then pulled out. Categorising of the ovaries followed the method described by Saunders (302) described in section 3.1.3.
Having been aged, the female flies then had their midguts dissected out by removing the abdomen from the thorax and drawing out the midgut. The midgut was then macerated with the dissecting forceps and screened using one of the two compound microscopes at 200x and 400x using the dark-field filter in order to check for trypanosomes. The salivary glands were left intact and screened at the same magnification as the midgut tissue. Finally, the mouthparts were removed and dissected separately on the slide and again screened for infection as the midguts and salivary glands were screened.

A tissue was deemed infected if living trypanosomes were seen within the tissue and negative tissues were devoid of any visibly motile trypanosomes. All tissues were stored in 60µl of 100% ethanol (ETOH) including those that were trypanosome negative using traditional microscopy methods. The tissues from each fly were stored separately on a 96 well PCR plate sealed with strip caps, so that each plate comprised 32 flies divided up into mouth parts, salivary glands and midgut.

3.2.3 DNA extraction

A subsample of 2,184 of the 6,664 dissected tsetse were selected for further analysis using the mITS primers described in Chapter 2 (section 2.2.6). Prior to extraction of DNA tissues from each fly were preserved separately in 60µL of 100% ethanol. The DNA extraction was carried out on each individually preserved tissue so that for each tsetse its midgut, salivary glands and mouthparts would have their DNA extracted and stored. The DNA extraction followed the protocol described in Chapter 2 section 2.2.2.

3.2.4 Trypanosome PCR

The tsetse samples were to be processed with the generic ITS primers however the presence of a *Bodo sl* contamination cross reacted with ITS primers producing a product of similar size to *T. vivax*. This led to the development and adoption of the mITS primers for all work in this chapter as the specificity of the mITS primers resulted in no cross reaction with *Bodo s.l.*. The analysis of the tsetse samples followed a two-step process. First, 2µL aliquots of the different tissues for each fly were pooled together and these were screened with the mITS for any positive tsetse. Second, upon identifying any positive pools of tissues the midgut, salivary glands and mouthparts from the positive pool were then re-screened individually to identify which organ of the tsetse was infected, and therefore to determine if the infections were mature or immature. The samples identified as positive for *T. brucei* s.l. would be screened with the *T. brucei s.l.* multiplex primers designed by Kim Picozzi (271) and with the TgsGP primers specific for *T. brucei gambiense (272)*. The *T. brucei s.l.* multiplex included universal *Trypanozoon* primers that target a single copy gene, phospholipase C (PLC), (330-332) and primers that amplify the serum resistance associated (SRA) gene. Amplification of the PLC gene indicates that there is enough material in the template for the detection of single copy genes, such as the *T. b. gambiense* specific glycoprotein (TgsGP) primers. The mITS PCR and *T. brucei* sub-species PCR followed the methods described in Chapter 2 (section 2.2.5).

3.2.5 Identification of T. brucei s.l. sub-species

There is a difference between the sensitivity of the primers used to identify samples positive for *T. brucei s.l.* and those used to identify down to the sub-species level. The Tbr primer

targets a copy region of several thousand (267) and the mITS primers target a ~200 copy region (333) whereas the *T. b. rhodesiense* and *T. b. gambiense* species specific primers target a single copy region of the genome. Consequently, it may be that while samples can be unequivocally identified as being from *T. brucei s.l.* the quality and/or quantity of the DNA extracted is insufficient to identify the sub-species. Accordingly, the following methodology was used to quantify whether samples contained sufficient DNA to allow the TgsGP primers to detect presence of genomic material from *T. b. gambiense* unequivocally.

Having identified the Tbr positive samples the same samples were then screened with the *T. brucei s.l.* multiplex primers designed by Kim Picozzi (271) and with the TgsGP primers specific for *T. brucei gambiense (272)*. The *T. brucei s.l.* multiplex included universal *Trypanozoon* primers that target a single copy gene, phospholipase C (PLC), (330-332) and primers that amplify the serum resistance associated (SRA) gene. Amplification of the PLC gene indicates that there is enough material in the template for the detection of single copy genes, such as the *T. b. gambiense* specific glycoprotein (TgsGP) primers. The primers used in the *T. brucei s.l.* multiplex are shown in Table 6.1.. Primers 657 and 658 amplify a single copy *trypanozoon* PLC gene. The SRA gene is similar to variable surface glycoprotein (VSG) genes, to help distinguish SRA amplification from VSG amplification two primer sets were designed, which when run together, produced SRA products of 669bp and VSG products of >1Kb (271).

3.2.6 Blood meal analyses

Of the 2,184 tsetse that had undergone DNA extraction, 768 tsetse midguts were tested with a universal vertebrate cytochrome B primer to screen for host DNA (199). The 768 subsamples analysed comprised 384 tsetse from each of the wet (September 2013-November 2013) and dry (December 2013-February 2014) seasons and were selected randomly from tsetse samples processed in the middle period of each month (10^{th} to the 20^{th}). It was necessary to make a one in ten dilution of neat tsetse midgut DNA elution to act as the DNA template as some of the samples had a large blood meal that inhibited the PCR reaction, either through over saturation of DNA or due to a higher proportion of inhibitors in the blood.

3.2.7 Weather data

The importance of the weather on tsetse populations has been critical in understanding tsetse ecology and distribution (334-336). The knowledge generated by such studies has contributed to the development of various predictive models designed to assess the effects of weather changes on tsetse populations (337).

To better understand the data generated by this study it is essential to relate the tsetse data to meteorological data. As local weather data were unavailable, rainfall estimates (rfe) were derived from the tropical applications of meteorology using satellite data (TAMSAT) online model developed by the meteorological department of Reading University (338). A rectangular area of Koboko (N:3.8, W:30.8, E:31.5 and S:3.2)(338) was used as the area of interest for the model, this area covered the study area of Koboko. Alongside rfe the maximum, minimum and average temperatures for each month were obtained from climate-data.org (339). The monthly temperatures were average temperatures generated from a climate model with 220 million global data points from 1982-2012.

3.2.8 General statistical methods

All statistical analyses were carried out using R (340). Catches (n) were not Normally distributed and so the use of classical statistical tests and standard estimates of means and error estimates based on an assumption of a Normal Distribution are inappropriate. Accordingly, a number of approaches were therefore adopted. First, to provide simple estimates of relative abundance the median catches and their respective inter-quartile ranges and the 5% and 95% centiles are reported. Second, to analyse the statistical significance of differences in the catch of tsetse, catches were Normalised using a $\log_{10}(n+1)$ transformation and then subjected to an Analysis of Variance (ANOVA). Third, in some cases raw (i.e., not transformed) catch data were analysed using Generalized Linear Models (GLM) or Generalized Linear Mixed Models (GLMM, using the glmmADMB package) with either a Poisson or Negative Binomial error structure. Several types of data comprised proportions. For example, the proportion of the catch that was a particular sex (male or female), various ovarian categories (0-7) or infected with trypanosomes. These data are also not Normally distributed and were therefore analysed using a GLM or GLMM with a Binomial error structure. For instance, to analyse differences in the proportion of males (or

females) in a catch, the number of males was specified as the response variable and the total catch (males+females) as the Binomial denominator. Further details of the statistical methods and models as applied to particular types of data are reported in the Results section below.

3.3 Results

3.3.1 Tsetse catch data

Across the sampling period from April 2013 to July 2014, traps were deployed at the four sampling sites for 287 days and a total of 12,532 tsetse with 930, 2857, 3543 and 5092 tsetse caught at trap sites KO16, KO19, KO20 and KO21, respectively. The median daily catch of tsetse caught from the four trap sites, varied between a peak of 14 in November 2013 and a low of 4.5 in April 2013. To assess whether there was a significant difference in catch between months, a GLMM was fitted to the data with sites and days specified as random effects and months as a fixed effect. The best fit to the data, as judged by having the lowest Akaike information criterion (AIC), was obtained by specifying a Negative Binomial error structure with zero inflation. There was a significant (Deviance=84.8, df=15, $P<0.001$) effect of month on the catch. Pairwise comparison of the catches (Tukey multicomparison test) showed that there were significant differences between, on the one hand, months (April 2013; January, February and April 2014) with relatively low catches (5.0-7.4 tsetse/day) and November-December 2013 when the mean daily catches ranged between 19.4 and 17.9 tsetse/trap (Fig. 3.6).

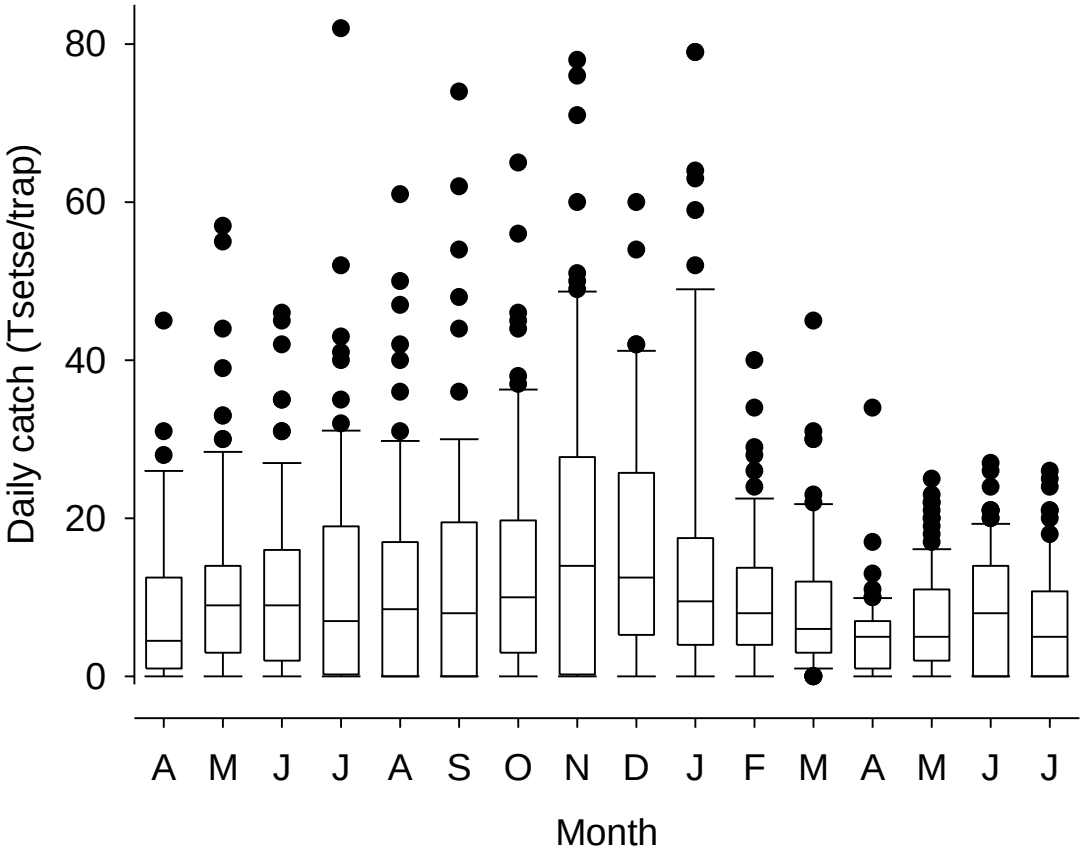


Figure 3.6 Median catch of tsetse per month between April 2013 and July 2014
males+females pooled (boxes=interquartile range, whiskers=10 and 90 centiles). In this figure the outliers are represented by the black dots.

The relationship between male and female tsetse was studied by analysing the variation in proportion of females across the year. Overall, 78.% (9749/12422) of the catch was female and there was no clear or significant variation in the proportion of females (Fig. 3.7B) between months (Tukey multiple comparison test).

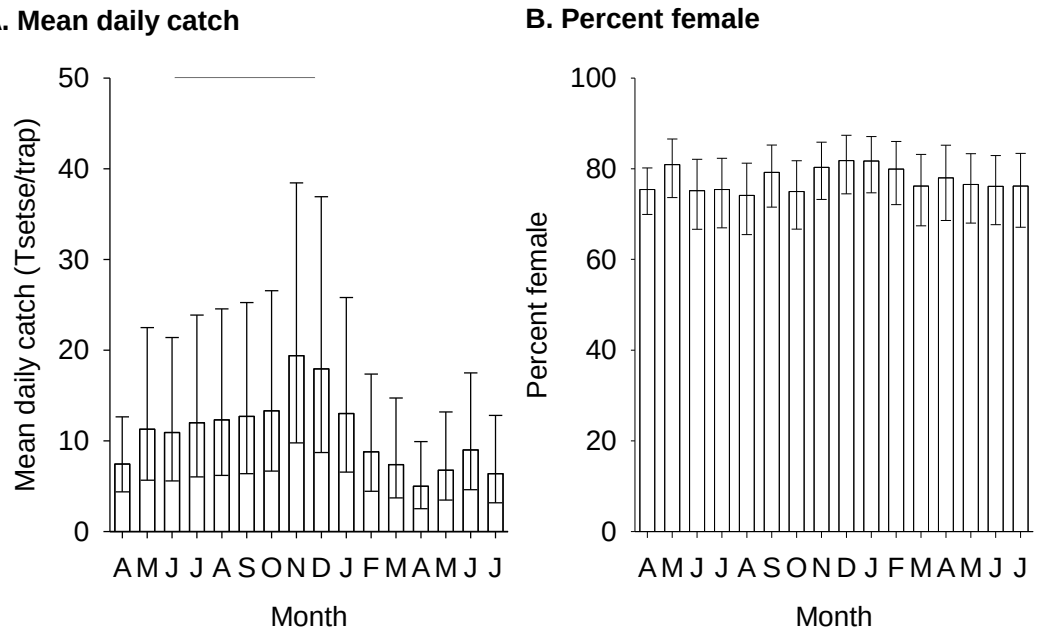


Figure 3.7 Mean tsetse daily catch and female percent

(A) Mean (±95%CI) daily number of tsetse and (B) percentage female caught from traps operated between April 2013 and July 2014

3.3.2 Interaction between catch numbers, temperature and rainfall estimates

To assess the potential causes of the intra-annual variation in the apparent density of tsetse (Fig. 3.7A), the effects of temperature and rainfall on the catch of tsetse was assessed by regression analysis of the mean for each month (Fig. 3.7A) against monthly rainfall and temperature. There was no significant effect of temperature (Fig. 3.8) but analyses of the rainfall data (Fig. 3.9) showed that while there was also no significant effect of rainfall during the month that the tsetse were collected, there was a significant relationship with the rainfall in previous months. In particular, the rainfall in one month (*m*) was a stronger predicter of relative abundance three months later (*m*+3). For instance, there was no significant correlation (r^2=0.002, Fig. 3.9A) between catch in a month and the rainfall for that month whereas there was a strong correlation (r^2=0.618, Fig. 3.9D) between catch and the rainfall three months previously.

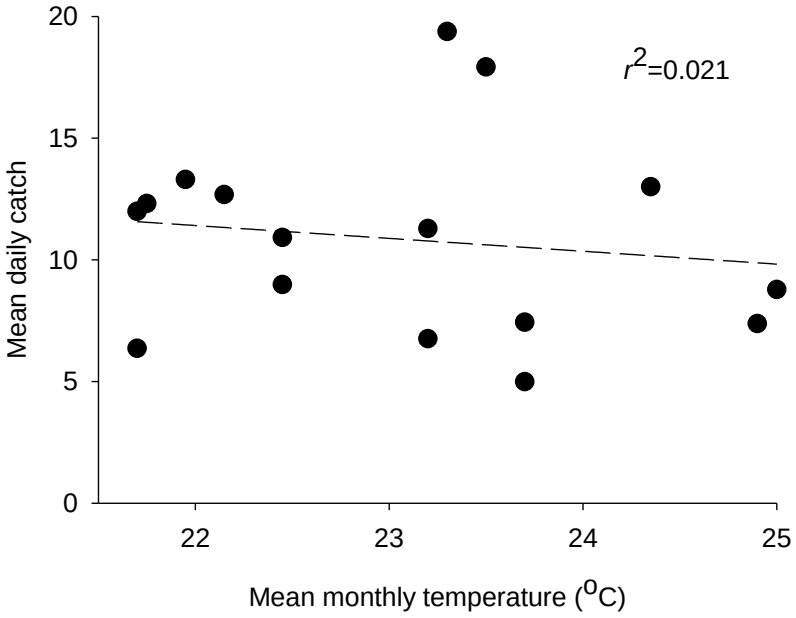


Figure 3.8 Scatterplot of mean daily catch against mean monthly temperature:

Dotted line indicates regression line (not significant)

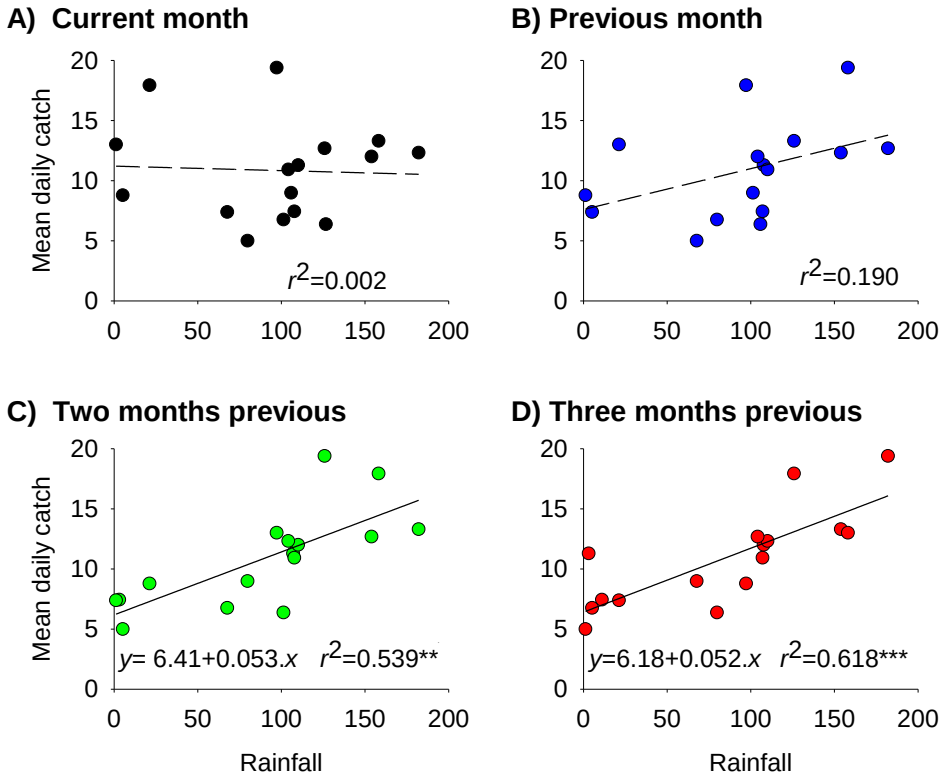


Figure 3.9 Scatterplot of mean daily catch for that month against: Scatterplot of mean daily catch for that month against:

(A) that month or (B) 1, (C) 2 or (D) 3 months previously. Solid and dotted lines indicate significant and non-significant regression lines respectively.

3.3.3 Age structure

The distribution of age categories was not Normally distributed. To assess the significance of changes in age structure, the proportion of tsetse old enough to have developed a mature infection of trypanosomes (Ovarian category >2) was calculated for each month. The results (Fig. 3.10) showed that there was a significant decline in the proportion of mature flies from a mean of 79% in April 2013 to 54% in July 2014. This change in age structure is likely to have an impact on the overall prevalence of trypanosome infections in the tsetse population since the proportion of tsetse old enough to harbour a mature infection was lower during the dry season (Jan-Feb 2014) and highest during the wet season (May 2013, May 2014).

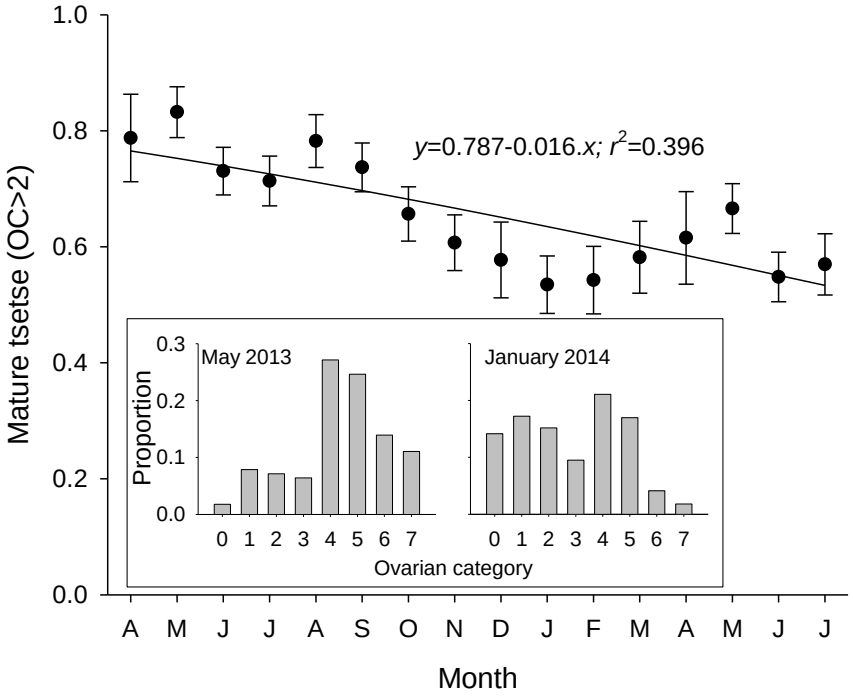


Figure 3.10 Proportion of 'mature' female tsetse for each month
Inset graphs show age structure for months when the proportion of mature flies was the greatest (May 2013) or smallest (January 2014).

3.3.4 Trypanosome prevalence

Of the 12,532 tsetse caught 6,664 (53%) were dissected, of which 89 (1.34%) were midgut positive, seven (0.11%) were salivary gland positive and 84 (1.26%) were mouthpart

positive. A sub sample of 2,184 tsetse, from September 2013 to February 2014, were then screened using the mITS primers to identify tsetse positive for *T. brucei s.l., T. congolense* and *T. vivax.* In comparison to the classical dissection results for these samples the PCR detected three times as many midgut positives, seven times as many salivary gland positives and twice as many mouthpart positives (Fig.3.11). The PCR assay consistently detected a higher number of positive tsetse across the sampling period than the traditional microscopy method (Fig 3.11), there were a total of 106, 34, and 64 midgut, salivary glands and mouthpart positives, respectively.

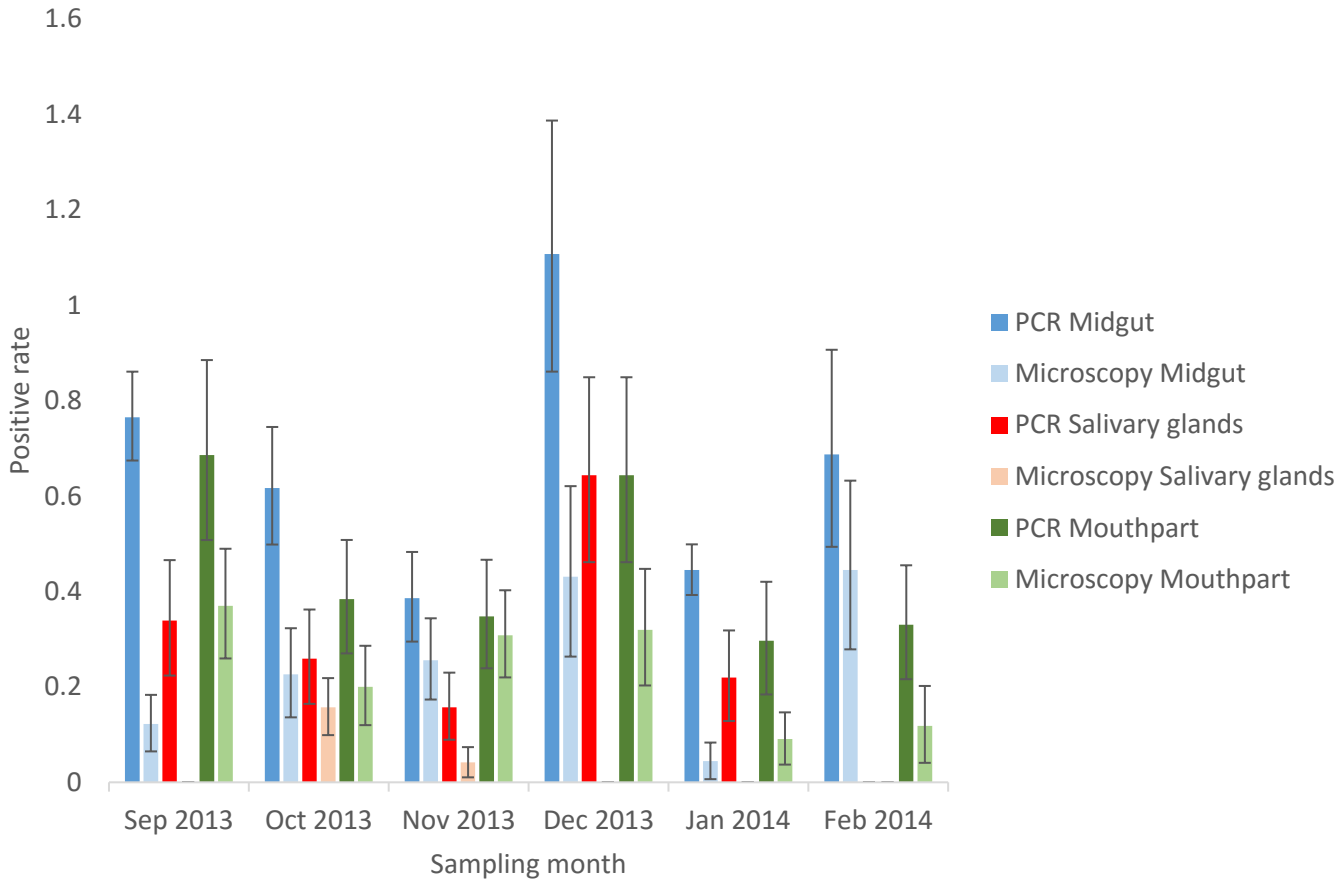


Figure 3.11 Comparison of microscopy and PCR positive results
The PCR method has been able to identify a greater number of samples positive for salivarian trypanosomes than the microscopy methods employed.

3.3.4.1 Comparison of microscopy and qPCR results

Mircoscopic examination of the 2,184 tsetse samples screened with the mITS PCR primers showed that 62 tissues were infected comprising: 30 midguts, 4 salivary glands and 28 mouth parts. The mITS primers identified infections in 48 of the 62 tissues detected by microscopy, Table 3.3.

Table 3-3 ***The mITS results of the microscopy positives***

		Midgut	Salivary glands	Mouth parts
	Microscopy positive	30	4	28
mITS results	***T. brucei s.l.***	4	2	0
	T. congolense	10	1	7
	T. vivax	0	0	15
	T. brucei s.l. +T. vivax	0	0	1
	T. brucei s.l.+T. congolense	6	1	1
	No ID	10	0	4

The mITS assay was used to screen 2, 184 tsetse flies, the initial screening of pooled samples identified 173 trypanosome positive tsetse. The midguts, salivary glands and mouthparts of these positive tsetse were then re-screened individually to determine which tissues were positive. Of the 173 pooled samples that were positive, it was possible to identify the individual positive tissues in 125 (75%). Of the three tissue types, midguts produced the greatest number of positives followed by mouthparts and then salivary glands with 106, 64 and 34 positives respectively. *T. congolense* was identified as the most prevalent trypanosome with 81/125 (64%) positive tissues, followed by *T. vivax* with 72/125 (58%) and *T. brucei s.l.* proved to be the least common with only 46/125 (37%) positive tissues. There were also five positive samples that produced a PCR product of a size that did not correlate with the three species of interest (Table 3.4). For all three of the species of interest there were instances where tissues not associated with their life cycle produced positive results. For *T. vivax*, there were four salivary gland positives and 34 midgut positives, *T. congolense* produced 16 positive salivary glands and *T. brucei s.l.* produced 10 positive mouthparts.

Table 3-4 ***The tissue positives for T. vivax, T. brucei s.l., T. congolense and unknown***

	Single tissue positives		
	Midgut	**Salivary glands**	**Mouthparts**
T. vivax	9	2	10
T. brucei s.l.	19	9	8
T. congolense	31	3	6
Unknown	3	0	0

	Multiple tissue positives			
	Midgut/mouthparts	**Midgut/salivary glands**	**Salivary glands/mouthparts**	**Midgut/salivary glands/mouthparts**
T. vivax	23	1	0	1
T. brucei s.l.	0	2	0	2
T. congolense	4	4	2	7
Unknown	0	0	1	0

Detection of trypanosomes in particular tissues is the classical method of distinguishing between the three main groups of salivarian trypanosomes, *Trypanozon, Nanomonas* and *Duttonela*. The advantage of microscopy analysis is that it allows for positive tissue and flies to be identified as live trypanosomes need to be observed. Of the 6,664 tsetse dissected and screened with microscopy 158 were positive for live trypanosomes in the three tissues screened. The prevalence of infection was greatest in older flies with OvC 6 producing the highest percentage of positive tsetse caught, (4%) Table 3.5

Table 3-5 ***The infection rates for all females by Ovarian categories, and males, per tissue type.***

	Infection numbers per ovarian category (OvC)										
	OvC 0	**OvC 1**	**OvC 2**	**OvC 3**	**OvC 4**	**OvC 5**	**OvC 6**	**OvC 7**	**Females**	**Males**	**Total**
Total tsetse caught	**505**	**812**	**565**	**437**	**1312**	**1098**	**424**	**254**	**5407**	**1257**	**6664**
Infected tsetse	**4**	**20**	**11**	**14**	**37**	**25**	**20**	**7**	**138**	**20**	**158**
Midgut	4	14	7	9	18	12	8	4	76	13	**89**
Salivary glands	0	0	0	0	3	1	2	0	6	1	**7**
Mouth parts	0	7	6	6	24	15	14	4	76	8	**84**
Total tissue positives	**4**	**21**	**13**	**15**	**45**	**28**	**24**	**8**	**158**	**22**	**180**

3.3.5 *T. brucei* species multiplex and TgsGP assay

3.3.5.1 *T. brucei* species multiplex

Of the 46 *T. brucei* s.l. positive tissue samples identified with the mITS primers 25 (56%) tested positively for the single PLC gene respectively. Of the 25 positive tsetse samples 13 were from midguts, 10 from the salivary glands and two were from the mouthparts. No samples were positive for the 669 bp sized band specific for the SRA gene which would indicate the presence of *T. b. rhodesiense*. The multiplex assay would produce different sized bands depending on the *T. brucei* s.l. sub-species. If there is sufficient genetic material for single gene copy amplification then all three sub-species would produce the phospholipase C band(PLC) (324bp); some strains could produce a VSG gene band (>1Kb); however only *T. b. rhodesiense* would produce the SRA band (669bp). In our study we found that our *T. b. gambiense* positive control and some of our field samples also produced a third band of >700bp in size. This band persisted despite increases in annealing temperature, Fig 3.12

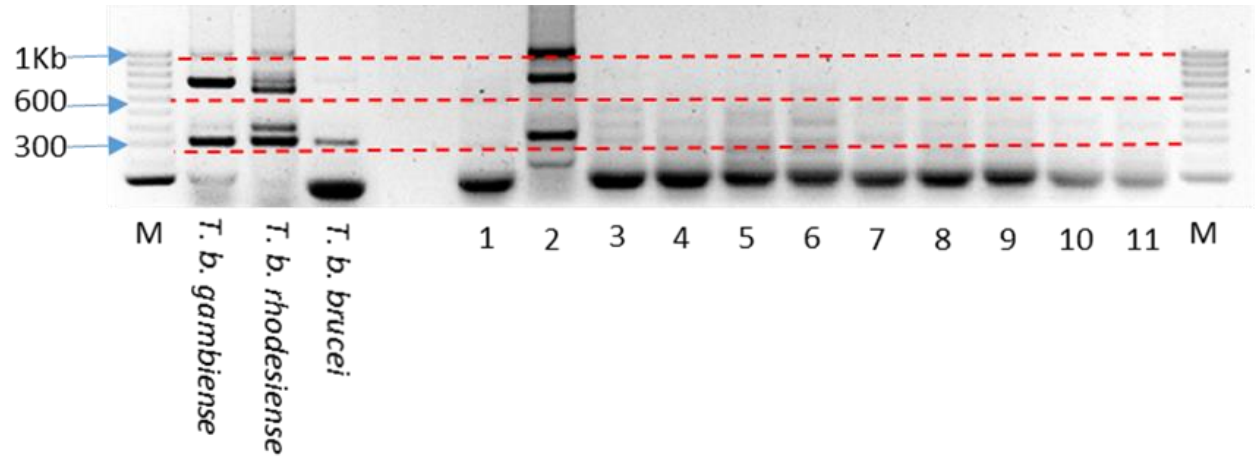


Figure 3.12 Representative results of the T. brucei s.l. multiplex assay
with positive controls for all three *T. brucei* sub-species. Samples 1-10 are tsetse midgut samples that were identified as positive for *T. brucei* s.l. using the mITS primer set. Sample 2 has a strong 324bp PLC band and a band >1Kb VSG band. This indicates that there is enough genetic material for the detection of a single copy gene. It is also easy to observe the difference in sizes between the SRA band at 669bp and the third band of approximately 700bp in size that appears in all three *T. brucei* s.l. sub-species, albeit faintly for *T. b. rhodesiense*. M denotes the molecular weight markers.

3.3.5.2 TgsGP assay

The samples were then tested with the TgsGP primers to screen for *T. brucei gambiense*. Of the cattle and pig samples none was positive for *T. b. gambiense* however eight midgut and eight salivary gland samples produced faint PCR products of approximately 308bp Fig.3.13.

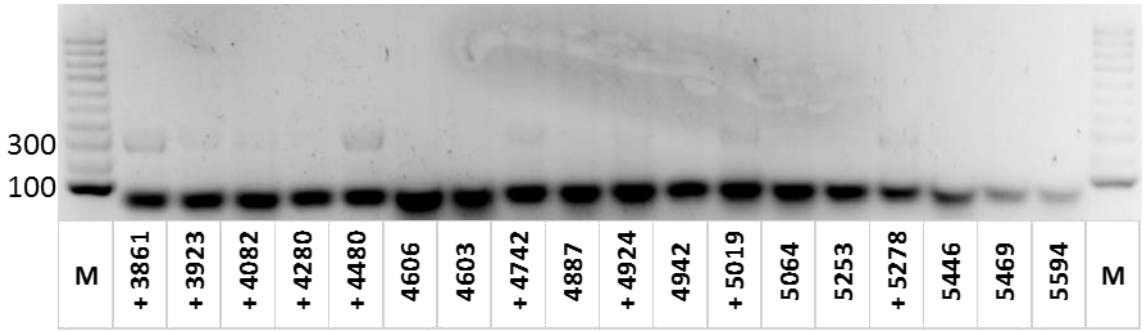


Figure 3.13 T. brucei s.l. positives
Results of tsetse samples processed with the TgsGP showing the presence of a ~308bp sized product after amplification with the TgsGP primers. The numbers given are the tissue sample reference numbers and '+' denotes a positive samples, the PCR ladders are labelled as M.

The bands produced by the TgsGP primers were very faint and only three bands from the midgut samples were sent for sequencing (samples 3861, 3923 and 4280) as these bands showed three different levels of brightness. The PCR products were purified using the ExoSap PCR clean-up kit. The samples were sent to SourceBioscience for sequencing using both the forward and reverse primers. The positive *T. b. gambiense* control was also purified and sent for sequencing.

3.3.5.3 TgsGP sequencing results

The sequencing results allowed for a more accurate measurement of the size of the products produced. The *T. b. gambiense* control product measured 308bp and was a 100% query cover and identity match with the *T. b. gambiense* sequences from the NCBI nucleotide collection data base. The three samples from the midgut produced a sequence that found no similarity when searched against the nucleotide collection database and measured 281bp. Fig.3.14 shows an alignment with the three sequenced midgut samples, the *T. b. gambiense* positive control and an NCBI blast reference sequence, accession number FN555990.

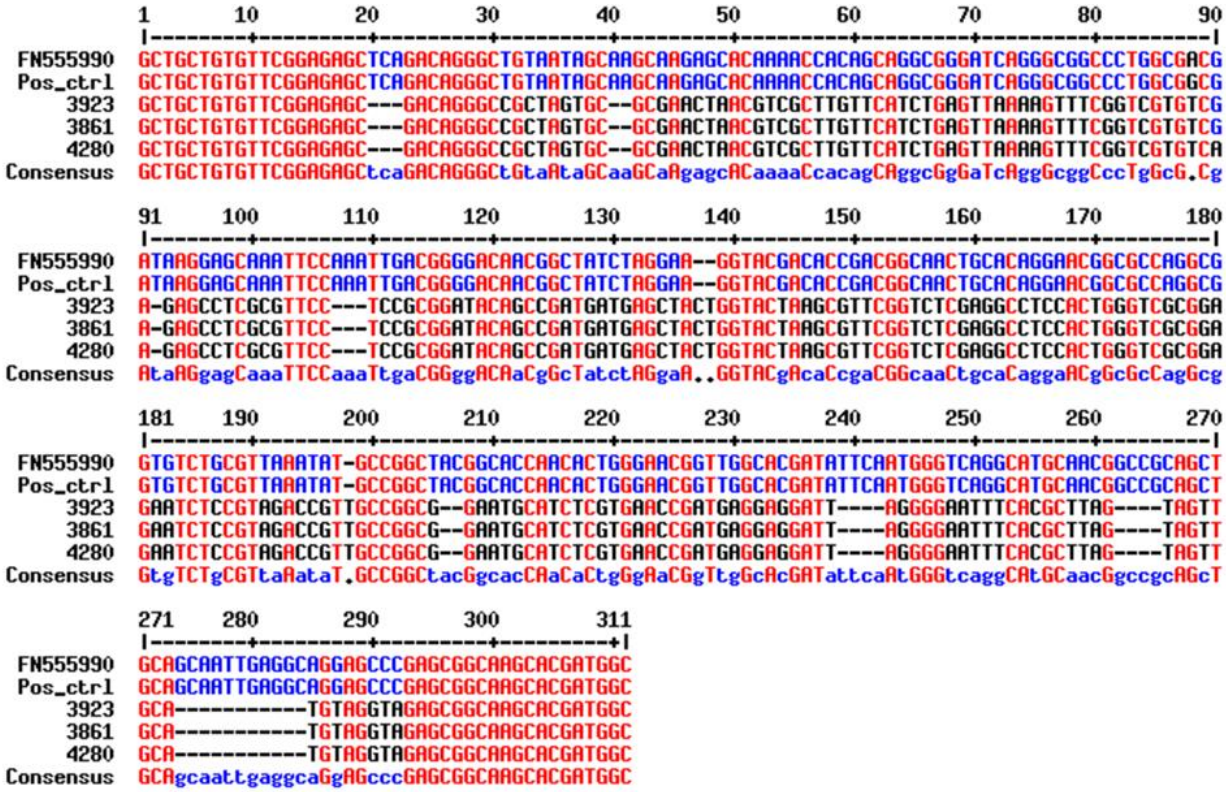


Figure 3.14 Aligment of the products generated by the TgsGP primers
These were aligned against the *T. b. gambiense* positive control and a reference sequence for the *T. b. gambiense* glycoprotein gene (accession number FN555990) from the NCBI nucleotide collection database.

3.3.6 Blood meal analysis

Following on from the molecular analysis targeting trypanosomes, a subsample of 384 flies were taken from the wet and dry season each, giving a total of 768 (36%) of the mITS screened tsetse. From these 768 tsetse, 131/768 (17%) blood meals were identified, 63 (48%) from the wet season and 68 (52%) from the dry season. The blood meals were comprised of the following host species: 48/131 (37%) *Homo sapiens* (humans), 51/131 (49%) *Bos taurus* (cow), *Varanus niloticus* (Nile monitor), 5/131 (4%) *Naja melanoleuca* (forest cobra), 2/131 (1.5%) *Capra hircus* (goat), 4/131 (3%) *Sus scrofa* (pig), 1/131 (0.8%) *Ovies aries* (sheep) and 1/131 (0.8%) unidentified species of passerine bird. Only the first four hosts were found in both the wet and the dry season the remaining host species were only identified in the dry season Fig 3.15.

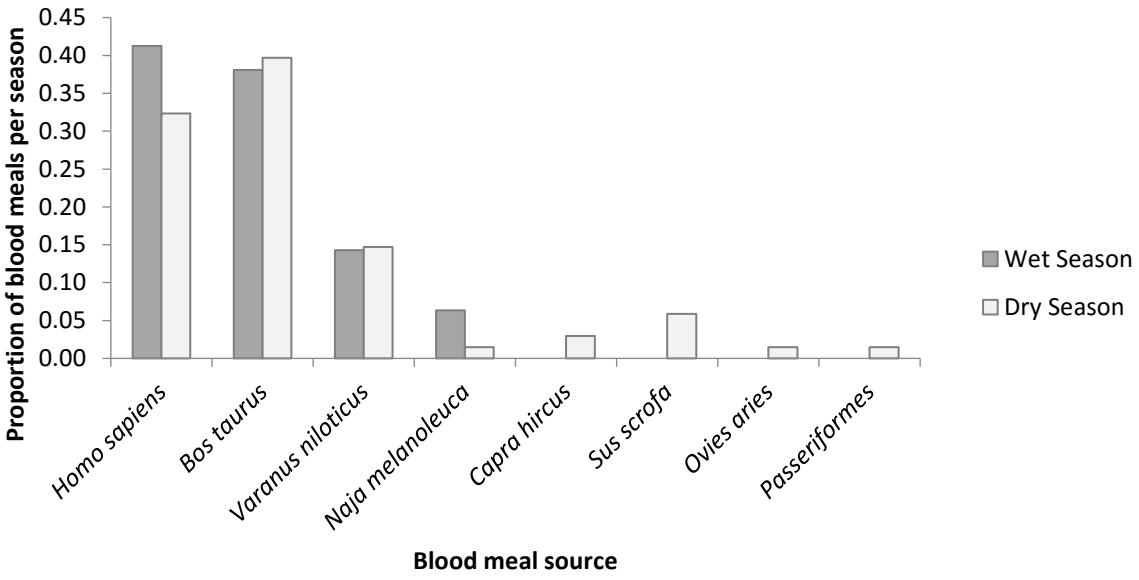


Figure 3.15 Blood meal identification from tsetse caught in wet and dry seasons
Of the 131 blood meals identified 63 (48%) came from the wet season and 68 (52%) came from the dry season.

The source of blood meals across the two seasons was analysed using the chi-squared test and found no significant difference between host feeding from the two seasons (*P*- 0.5), although the range of different host animals fed upon was greater in the dry season. In total eight different host species were identified by cytochrome B sequencing primers from the midguts of the tsetse screened. There was no statistical significance in the proportion of blood meals from humans and cattle in the wet versus dry season. The next biggest source of blood meals was taken from the Nile monitor (Fig 3.16).

3.4 Discussion

3.4.1 Tsetse population

3.4.1.1 Tsetse catch numbers

The number of tsetse caught along the Kochi from all traps was 13,876, averaging 867 flies per month, the male and female catch numbers differed significantly with the ratio of females to males being 3.6:1, but this ratio remained stable through the sampling period. This difference in numbers between the sexes may not be an accurate reflection of the sex ratio of the population as Fisher proposed that in a randomly mating population an equal sex ratio will be maintained (341). The differences in the numbers of males and females may reflect a sampling bias and/or differences in mortality rates. It is likely that both are true as the mortality rates in males have been estimated to be higher than females (342) and previous studies have shown that different tsetse catch methods attract male and female tsetse in different quantities. For example more males than females are caught with 'fly rounds' and the opposite is true with several designs of trap (343, 344). The number of tsetse caught did not vary markedly through most of the sampling period except for a dip two months after the end of the dry season in April 2014. This is in keeping with the finding that tsetse catch numbers were correlated with rainfall estimates 2-3 months prior to the catch date. Prior studies of tsetse populations have found a high degree of seasonal variability in the number of tsetse caught in traps (335, 345). The relative stability in tsetse catch numbers from our study site likely reflects the proximity of the study site to the equator and subsequent stable weather patterns compared to, say, the populations studied by Hargrove in Zimbabwe.

3.4.1.2 Tsetse age structure

The age structure of the tsetse population varied across the sampling period. Although there was no significant change in proportion of tsetse in different ovarian age categories in relation to rainfall, the average age of the tsetse population decreased during the dry season from December-February. There are no comparable studies of *G. fuscipes* populations. However, studies of *G. pallidipes* have shown an ***increase*** in the average age of the tsetse population and a drop in catch numbers during the dry season. It was concluded that this was due to an increase in the mortality of young tsetse and pupae (345). Present data shows the opposite, with an increase in the number of tsetse caught during the dry

season and a simultaneous decrease in the average age of the tsetse population. The reason for this difference could be that the higher catch numbers and younger flies are the result of a higher productivity of offspring during the end of the wet season. These larvae would be secure in the mother's uterus for two weeks and following deposition they would be protected within their puparium buried in the soil for a further three to four weeks. If during the rainy season there were less environmental pressures on the female population then it would follow that a greater number of larvae would be deposited only to emerge weeks later during the dry season. It has also been speculated that during dry seasons the frequency of feeds increases due to a shorter larval period and this increase in feeding rates brings with it an increased risk of mortality (346). Therefore, during the dry season there could be a large number of teneral flies emerging and at the same time older flies are dying off due to increased feeding, causing a lowering of the average age of tsetse catches.

3.4.2 Trypanosome positive tsetse

3.4.2.1 Dissection results

Microscopic examination of tsetse showed that 158 (2.5%) were infected with at least one species of *Trypanosoma*, which is lower than previous microscopy examinations of wild tsetse, which ranged from 5 to 17% (147, 259, 347). However the number of salivary gland positives, indicative of a mature *T. brucei s.l.* is in keeping with previous studies (135, 203).

Analysis of microscopy positive tsetse in Table 8 showed that the females with the highest infection rates were in ovarian categories 3, 6 and 6 for midgut, salivary glands and mouth parts respectively. Infected salivary glands were only found in tsetse in 4-6 in accordance with previous studies showing that older tsetse are more significant in the active transmission of salivarian trypanosomes (161). The traditional microscope-based method for the identification of trypanosome species based on infected tsetse organs (135) closely matched the results from the same samples screened with the mITS primers. Of the 2,184 tsetse screened with PCR, 62 (2.8%) were microscopy positive. The microscopy positives comprised of 30 midgut, four salivary gland and 28 mouthpart positives. The Lloyd and Johnson method would identify the midgut positives as immature infections of either *T. congolense* or *T. brucei s.l.*, the salivary gland infections as a mature *T. brucei s.l.* infection

and mouthpart infections as being either a mature *T. congolense* infection or a *T. vivax* infection.

The PCR-based method identified 48 (77.4%) of the microscopy positive samples, 21 (66.7%) from the midgut, four (100%) from the salivary glands and 24 (85.7%) from the mouthparts. The lower performance of the midgut samples is most likely down to an increased number of inhibitors in this tissue type as has been found in previous studies (348, 349). The 21 PCR positive midgut samples comprised 10 *T. congolense,* four *T. brucei s.l.* and six mixed positives of *T. brucei s.l.* and *T. congolense*. Of the four positive salivary glands, the PCR-based method identified two as single *T. brucei s.l.,* one mixed *T. brucei s.l.* and *T. congolense* and the remaining salivary gland positive was identified as a single *T. congolense* infection. *T. vivax* was not identified in either the microscopy positive salivary glands or midguts by PCR. The 24 PCR positive mouthparts consisted of 15 single *T. vivax* positives, seven single *T. congolense positives*, a mixed positive of *T. vivax* and *T. brucei s.l.* and a mixed positive of *T. congolense* and *T. brucei s.l.*. The congruence of the PCR results with what would be expected with the Lloyd and Johnson method is 100% for the 20 midgut positives and 91.7% for the mouthpart results. The salivary gland results deviated more significantly from the expected results with only 2 of the 4 microscopy positives being identified as single *T. brucei s.l.* infections. The final tally of PCR results that correlated with the traditional method for trypanosome identification in tsetse was 44 (91.7%) out of 48.

3.4.2.2 mITS PCR results

A total of 6,547 tissues were screened with the mITS PCR (2,183 midguts, 2,181 salivary glands and 2,183 mouthparts) first as pooled samples where the three tissue types for each fly were screened together and then later individually. The pooled samples detected 194 (8.9%) infected tsetse. When individual tissues of infected tsetse were screened, only 126 (5.6%) tsetse were found to have at least one tissue positive for either *T. brucei s.l., T. congolense* or *T. vivax*. The pooled samples produced 3x as many positives as microscopy while the individual tissue screening identified 2x as many positives as microscopy. The midgut samples produced 106 positives with 46 *T. congolense*, 34 *T. vivax* and 23 *T. brucei s.l.* plus three unknown products. The mouthparts contributed 64 positive results comprised of 19 *T. congolense,* 34 *T. vivax* and 10 *T. brucei s.l.* positives and one unknown. The salivary

glands produced 34 positive samples comprising of 16 *T. congolense*, four *T. vivax* and 13 *T. brucei s.l.* plus one unknown.

Single *T. brucei* and *T. congolense* positives were mainly identified in tsetse midguts, single salivary gland positives were predominantly *T. brucei s.l.* and single mouth part positives were predominantly *T. vivax*, with *T. brucei s.l.* and *T. congolense* occurring in near equal numbers. The correlation between different tissue positives per trypanosome species, Table 3.4, showed a *T. vivax* positives had a strong correlation between positive midgut and mouthparts. This is most likely due to trypanosomes from an infected mouth part being passed into the midgut during feeds resulting not in a midgut infection but rather the trace DNA of these parasites being picked up by the PCR. The high number of *T. congolense* salivary gland results are at odds with the known developmental cycle of the parasite. This could be explained by the dissection method employed, if the mouthparts are gripped prior to removal of salivary gland it is possible that saliva and trypanosomes could be forced back into the salivary glands. Alternatively, the development of *T. congolense* involves a stage in the cibarium, and here the salivary glands meet the mouthparts, so it is possible that the salivary gland could have *T. congolense* at the apical end. A limitation of the PCR method compared to microscopy is that it is unable to determine whether these positives are an active infection or relic DNA left over from dead trypanosomes that failed to establish an infection. This is especially true of midgut samples where trypanosomes could be taken up with a blood meal, including non-salivarian species.

3.4.2.3 Identification of T. b. gambiense in local tsetse

When screened with the *Trypanozoon* sub-species multiplex assay, 56% of the Tbr-FIND positive tsetse samples reacted successfully, indicating sufficient DNA being present for single-copy gene detection. Of these samples, 16 tsetse produced faint bands of approximately 308bp in size. A subset of these were then sent for sequencing alongside a *T. b. gambiense* positive control. The sequencing results showed that the size of the product generated by the samples was smaller than the expected size at only 281bp compared to the expected 308bp sized product. There was also significant variation in the 281bp sized sequences compared to *T. b. gambiense* sequence. Based on the sequencing results these positive samples cannot be considered to be *T. b. gambiense* and without further analysis it

is not possible to determine the origin of the bands produced by the TgsGP primer set as the sequence failed to match any known sequence in the NCBI databases. What can be said is that of the 25 tsetse samples that possessed enough genomic material to amplify single copy genes none was found to be positive for *T. b. gambiense*. This lack of positive samples reflects the overall low prevalence of the disease and the continued decrease in the number of cases in Uganda (350). Despite not finding *T. b. gambiense* in the tsetse population of Koboko vector control has been calculated to being essential to reach the elimination goal of 2020 (351).

3.4.3 Bloodmeal analyses

The analysis of the bloodmeal results show no differences across the two seasons between the top three sources of bloodmeal, cattle, human and Nile monitor lizard. The biggest difference in the feeding behaviour in the dry and wet seasons was that a greater variety of animals were fed upon during the dry season than in the wet season. Comparing the bloodmeal results with the data in table 3.2 shows that despite chickens and goats being in greater numbers cattle, nile monitor and forest cobra are fed upon more. Similarly, despite being the least common domestic animal pigs were fed upon in greater numbers than either sheep or goats which occur in 80x and 250x greater numbers. Other studies looking at the feeding habits of *G. f. fuscipes* found that cattle comprised 30%-40% of total blood meals (352), this study has a similar level of cattle blood meals comprising 39%. However, in this study levels of human blood meals were close the those of cattle at 37% which is far higher than those of the Waiswa study and is probably related to the fact that the area in this study is a gHAT foci.

3.4.4 Conclusions

Overall the tsetse catch numbers remain stable during the wet season and drops in the dry season. This probably relates to the relative stability of the equatorial climate compared to the more extreme climate of tsetse-infested areas of southern Africa. While the fluctuation in numbers is relatively slight, there is some intra-annual variation in apparent density and this seems to be related to rainfall in preceding months. There was also a significant variation in the age structure of the population during the 16-month course of the study.

These changes in tsetse numbers and age structure will affect the transmission potential of the tsetse population.

Microscope and PCR-based analyses of trypanosome infection in tsetse showed that both methods identified *T. brucei s.l., T. congolense* and *T. vivax* positive tsetse. However, the PCR-based method had a higher sensitivity and identified three times as many positives as the microscopy method. The finding that *T. brucei s.l.* was only detected in older tsetse (Ovarian category 2) is in accordance with expectation and underlines the likely impact of changes in the age structure of tsetse populations on transmission of this species of *Trypanosoma*.

Lastly and most importantly, the study identified salivary gland positive tsetse both through microscopy and PCR, implying the active transmission of *T. brucei s.l.* species in Koboko. The blood meal analysis helps to identify the most likely hosts of *T. brucei s.l., T. coongolense* and *T. vivax* in the area with a heavy predation of cattle. Despite the area being a predominantly Muslim, pigs were also identified as a blood meal source and recent studies have suggested pigs may serve as a reservoir for *Gambiense* HAT. It would therefore be worthwhile sampling members of the pig population in NW Uganda when searching for *T. b. gambiense*. These data suggest that potential hosts of *T. brucei* are humans, cattle and pigs.

Chapter 4 : Loop-mediated isothermal Amplification (LAMP) for field based xenomonitoring

4.1 Introduction

4.1.1 Background

Detection of *Gambiense* HAT relies largely on active or passive screening of the human population at risk. Active screening is difficult; screening >70% of a target population is seldom achieved (353) and the most common method of active screening, the card agglutination test for trypanosomiasis (CATT)(138) is insufficiently specific <96% (354). This lack of specificity means that the CATT assay is followed up by a series of tests including lymph node puncture, fresh blood and thick blood film examination (355-357). The sensitivity of the other tests vary as they use microscopical examination and therefore depend on the skill of the technician (137). Therefore the sensitivity of lymph node aspirates varies from 40-80% and blood microscopy methods have a sensitivity that varies from 39-80% (53)

The CATT test also has a low positive predictive value when used for screening populations in which the prevalence of gHAT is low (354, 358, 359). This means that the current method of screening for sleeping sickness may become unsuitable as the prevalence of the disease continues to decrease, making elimination less likely. Moreover, as the prevalence of HAT decreases, the cost of detecting each case increases, leading to active screening programmes being scaled down or abandoned as other health priorities take precedent (246, 360, 361) An alternative to screening people is to screen vectors for the presence of the pathogen. This approach, termed xenomonitoring, has proven useful in the control of other vector transmitted diseases such as lymphatic filariasis (362). Rather than screening people, tsetse flies might be caught using simple traps and subsequently analysed for presence of *T. brucei s.l*.. By monitoring trypanosomes in tsetse, xenomonitoring would measure current rates of transmission in a defined area. By contrast, the chronic nature of *Gambiense* HAT and the mobility of humans mean that cases are detected months, if not years, after infection, and, potentially, in places far removed from sites of transmission (59).

4.1.3 Xenomonitoring of tsetse

There is a need for a simple yet highly sensitive and specific surveillance system for HAT, designing a xenomonitoring protocol that combined a simple DNA extraction method with a LAMP assay could fill this gap in resource poor settings (263, 363) . The screening of vectors for parasites is a standard practice in understanding disease transmission, however as shown in Chapter 3 the prevalence of different trypanosome species can be extremely low with *T. brucei s.l.* being the lowest. Through classical dissection techniques (135) the number of salivary glands found to be positive from the 6,664 tsetse dissected was seven (0.1%). The use of the mITS PCR assay had a higher sensitivity identifying 13 out of 2,587 (0.5%) salivary glands as *T. brucei s.l.* positive. Sceening based on use of microscopy or PCR require skilled technicians, specialised equipment and overheads (364). Out of the two methods the PCR is more costly in both time and money, however it is more sensitive and specific than the microscopy method (349). The advantages of LAMP over PCR is two-fold, first it is a relatively simple assay to perform, secondly it requires less resources than PCR such as it has no need for thermocyclers, gel electrophoresis and gel imaging equipment. LAMP assays are also very sensitive and specific (365, 366). The LAMP test for HAT used in this study amplifies the repetitive insertion mobile element (RIME) of the *Trypanozoon* group (152).

Studies of trypanosome infection in tsetse flies are generally concerned with quantifying the prevalence of pathogens in the vector population. For this purpose, the status of infection in individual flies must be quantified; only tsetse with a mature infection where *T. brucei s.l.* are observed in the salivary glands are infectious (367). For xenomonitioring however, the aim is to detect the presence or absence of pathogens in a vector population. This specific aim offers two opportunities to improve the cost-effectiveness of screening. First, pooled groups rather than single individuals might be screened for the presence of pathogens. Second, a molecular method may be able to detect trypanosomes that have been recently ingested but which will not lead to a mature infection. Older tsetse flies are much less susceptible to infection with *T. brucei* (368). However, flies that are refractory to infection will nonetheless ingest trypanosomes from infected hosts and a sensitive method may be able to detect these transient trypanosomes in recently-fed flies (369).

4.1.2 Loop-mediated isothermal amplification (LAMP)

LAMP is carried out at a constant temperature, thereby removing the need for thermocyclers, and results are read visually (150). The LAMP reaction uses a series of six primers that produce a DNA product with multiple annealing sites and results in continuous target DNA replication. The reaction is so efficient that the amount of DNA produced changes the turbidity of the reaction, causing the reaction mix to change from clear to cloudy. It is therefore possible to use this as a means of assessing a positive reaction without the need for post reaction processes such as gel electrophoresis. A diagrammatic representation of the LAMP reaction is depicted in Fig 4.1.

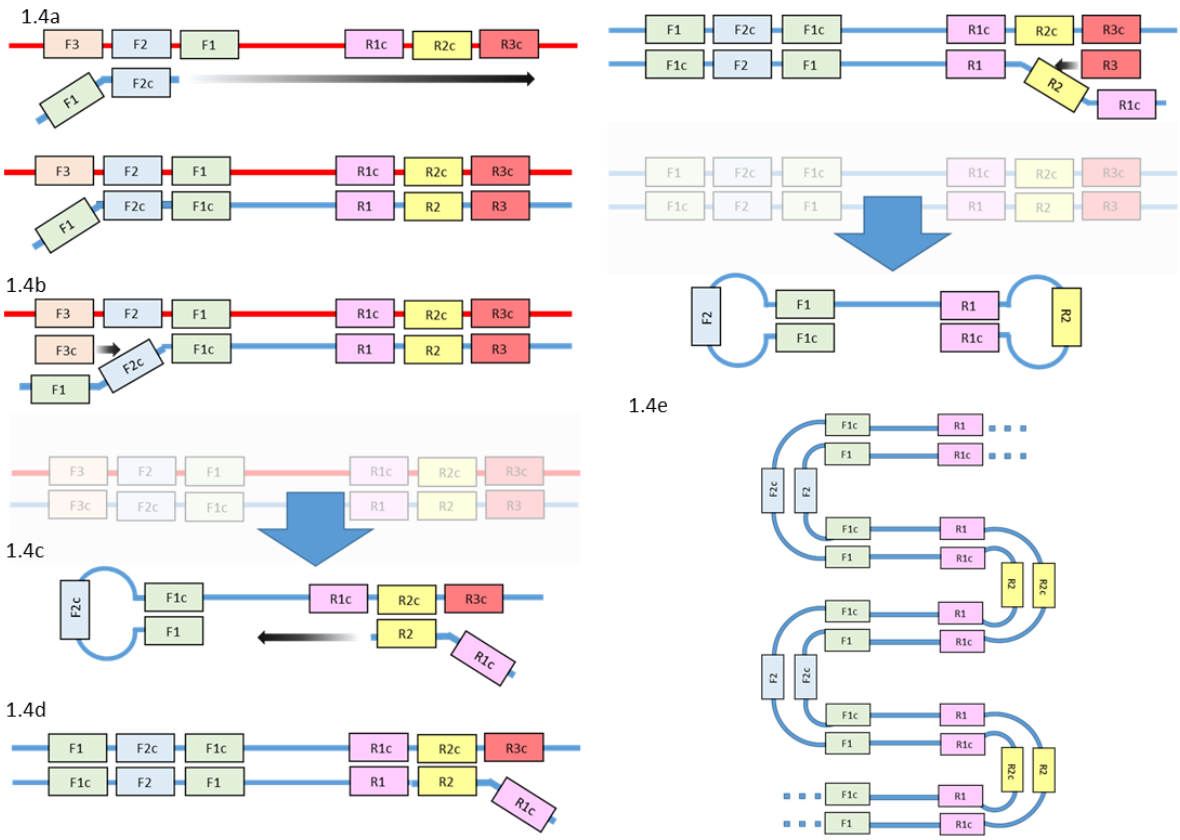


Figure 4.1 ***Depiction of the loop-mediated isothermal amplification (LAMP) reaction***
The LAMP assay uses six primer pairs specific to the target DNA sequence, which include inner and outer primer sets. 4.1a Amplification initiates from strand invasion from one of the inner primers, strand displacing DNA polymerase then extends the primer and separates the template DNA duplex. 4.1b The first product is then displaced by the synthesis of a secondary product initiating from an outer primer located on an upstream target region. 4.1c As the first product is displaced by the DNA polymerase it forms a self-hybridising loop structure, similar to a hair pin, due to the inclusion of a reverse complementary sequence in the primer design. A reverse inner primer is able to anneal to the 3' end of the hairpin. 4.1d Cycles of annealing and displacement repeat from the opposite end of the target sequence on the hairpin structure resulting in a self-hybridising loop at both the 5' and 3' end, this dumbbell structure forms the seed for exponential LAMP amplification. 4.1e There are now multiple annealing sites located at the 3' end of the open loops for both the inner and loop primers. The amplification proceeds from these sites and the product grows repeating the target sequence into a long continuous DNA molecule. This concatemer has itself multiple annealing sites causing a rapid increase in double stranded DNA that far exceeds the amount of DNA produced in a PCR assay (370). Image adapted from (150).

4.1.4 Aims

Previous work has demonstrated the ability of LAMP to detect *T. brucei* in laboratory-infected tsetse and in pooled groups of tsetse (154) however these studies have not tested commercially available LAMP kits. The aims of this study were

1) Design a field friendly DNA extraction protocol
2) To assess the sensitivity of the commercial LAMP kits against pooled tsetse samples and dilution gradients
3) Determine how long after an infective blood meal can *T. brucei s.l.* DNA still be detected
4) Test the specificity of the LAMP kits against laboratory infected tsetse and also field caught tsetse

4.2 Methods

Tsetse samples were tested using the Loopamp™ kit to determine the presence of trypanosome DNA, and the limit of detection of LAMP. DNA extraction methods were optimised for field settings and tested with the LAMP kits in pooled fly assays. The specificity of the kit was tested against laboratory (*G. m. morsitans*) and wild tsetse (*G. f. fuscipes*) infected with non-target species of trypanosomes likely to co-infect tsetse in areas where *T. brucei s.l.* occurs.

4.2.1 DNA Extraction

Tsetse flies were dissected and their midguts stored in 60µl of 100% ethanol (EtOH). At the Liverpool School of Tropical Medicine (LSTM) a standard DNA extraction procedure, hereafter referred to as the Chelex method, was followed: 70µl of distilled water was added to the midgut sample followed by centrifugation at 13,000rpm (15sec) and removal of 100µl of supernatant. Tissue samples were washed three times by adding and removing 100µl of distilled water. To the washed tissue, a 100µl suspension of Chelex and Proteinase K (20mg/ml) was added to give a final concentration of 1% Chelex, and incubated at 56˚C for 1 h. The sample was then incubated for 30 minutes at 93˚C, centrifuged at 13,000rpm (15sec), the supernatant removed, and stored at -20C.

4.2.2 Simplification of DNA extraction

The current Chelex DNA extraction method, although robust, is not field-friendly and takes ~2 h to complete with a number of steps and components, some of which require a cold chain. To use this kit in the field, the current method needs to be optimised to ensure it is rapid, robust and simple. Five alternative DNA extraction methods aimed at reducing the time and complexity of the current Chelex extraction method for use in field studies were designed (Table 4.1).

Table 4-1 ***Details of five alternative field-friendly DNA extraction methods.***

Method	Spin 13,000 rpm	Wash x3	Spin 13,000 rpm	5 % Chelex or 1% TE	Proteinase K	Incubation		Total Time (Min)
						56˚C	93˚C	
Chelex	Y	Y	Y	Chelex	Y	60 m	30 m	120
1/2 time chelex	Y	Y	Y	Chelex	Y	30 m	15 m	50
1/4 time chelex	Y	Y	Y	Chelex	Y	15 m	7.5 m	30
TE wash off alcohol	Y	Y	Y	TE			15 m	45
TE with chelex	Y	*		Chelex			15 m	15
TE leave in alcohol	Y			TE			15 m	22

*The alcohol was not washed off, instead the samples were transferred out of the EtOH and into a clean empty tube

These different DNA extraction methods were tested against two sets of trypanosome concentrations at 10^2 and 10^4 trypanosomes per ml, with each of these concentrations being spiked into midguts and whole tsetse abdomens in four replicates of each concentration. In total each DNA extraction method would be tested against 16 samples, Table 4.2.

Table 4-2 Overview of samples used in assessment:
Showing the *number of samples spiked with different concentrations of trypanosomes to be processed with the different DNA extractions*

DNA extraction method	Concentration of trypanosomes per 100ul				Total number of samples
	10^2 Midgut	10^2 Abdomen	10^4 Midgut	10^4 Abdomen	
DNA extraction method	4	4	4	4	16
TE Leave in alcohol	4	4	4	4	16
TE wash off alcohol	4	4	4	4	16
Reduced Chelex	4	4	4	4	16
Chelex	4	4	4	4	16
TE with Chelex	4	4	4	4	16
Reduced Chelex, reduced time	4	4	4	4	16

The two concentrations of trypanosomes tested were produced by calculating the number of trypanosomes in a defrosted trypanosome stabilate using a haematocrit reader. The concentration of trypanosome in the stabilate was then used to calculate how much was required to be added to horse blood to produce the two final concentrations described above.

To determine which DNA extraction method was best suited to the field three factors were considered:

1. -Time- how long does each method takes to perform
2. -Steps- how many steps are involved in each method; this is used as a proxy for complexity
3. -Positives- the total number of positives identified

The ideal method would produce a high number of positive results in a short amount of time, with as few steps as possible, to determine this efficiency the following formula was used:

$$\frac{\text{Time}}{\text{Number of steps}} \text{ x Number of positives}$$

4.2.3 LAMP

The RIME LAMP test was performed using the Loopamp^{TM} *Trypanosoma brucei* Detection Kits manufactured by Eiken Chemical Co.,Ltd, Japan, according to the manufacturer's specifications. Briefly, 2.5µl of template DNA was added to 22.5 µl of nuclease-free water. LAMP reagents were reconstituted in the lids of the tubes, and after two minutes incubation, the tubes were inverted five times. A thermocycler was used to heat the samples for forty minutes at 65˚C and then at 85˚C for five minutes to stop the reaction. Results were determined by visualising presence or absence of fluorescence in the reaction tubes on a UV gel imager, and each sample was classed as positive or negative based on two separate blind screenings. A representative LAMP assay result is shown in Fig 4.2, this image shows a strip of eight sample tubes and a positive and negative control.

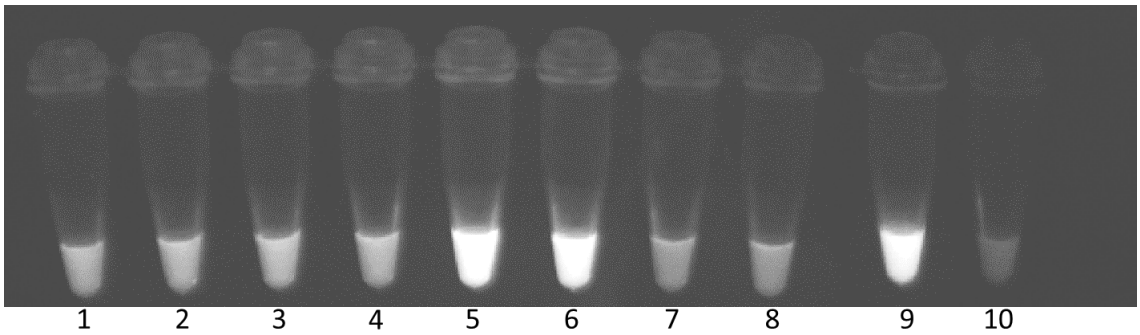


Figure 4.2 Representative LAMP assay result
This example shows eight samples (1-8) and a positive control (9) and negative control (10). Sample 5 and 6 are classed as positive while the remaining samples would be classed as negative.

4.2.4 PCR

Alongside the RIME LAMP assays, samples were also amplified using TBR primers(371) that target the same region of the gene as those designed by Moser(267) but are situated inside Moser's primers, thus producing a smaller product of 117bp compared with the original product of 177bp and a nested universal trypanosome ITS primer set(147) (Table 4. 3).

Table 4-3 Primers used in this study

Primer name	Target species	Primer sequence 5'-3'	Published/designed by
TBR-FIND Forward	*T. brucei s.l.*	TGCGCAGTTAACGCTATTATACA	Kazibwe 2008
TBR-FIND Reverse	*T. brucei s.l.*	AAAGAACAGCGTTGCAAACTT	Kazibwe 2008
Tryp 1	*Trypanosomatidae*	AAGCCAAGTCATCCATCG	Adams et al. 2006
Tryp 2	*Trypanosomatidae*	TAGAGGAAGCAAAAG	Adams et al. 2006
Tryp 3	*Trypanosomatidae*	TGCAATTATTGGTCGCGC	Adams et al. 2006
Tryp 4	*Trypanosomatidae*	CTTTGCTGCGTTCTT	Adams et al. 2006

The PCR reactions consisted of 12.5 µl MyTaq Red Mix (Bioline), 1 µl of forward and reverse primer (25mmol), 8.5 µl of nuclease free water and 2 µl of DNA template. For the TBR primers the PCR cycles were: an initial denaturation step at 93˚C for 2 mins, followed by 35 cycles at 94˚C for 10 s, 55˚C for 10 s and 72˚C for 10 s with a final extension step of 30 s at 72˚C, for the nested PCR 1 µl of PCR product from the first nest was added to the second to act as the template.

For both nests of the ITS primers the PCR cycle had an initial denaturation at 95˚C for 5 mins followed by 35 cycles of 94˚C for 15 s, 54˚C for 15 s and 72˚C for 10 s with a final extension step at 72˚C for 5 mins.

4.2.5 Limit of Detection

Colony-reared *G. m. morsitans* were dissected three days after their first blood meal, the midguts were collected into individual tubes and preserved in 100% EtOH. A trypanosome dilution series was created by first making a stock concentration of $2x10^5$ trypanosomes per ml using a haemocytometer (20). The trypanosomes were then heated at 93˚C for 30 mins to lyse the parasites and extract the DNA. Using the eluted DNA, a tenfold dilution series was created equivalent to $2x10^5$ trypanosomes/ml - 0.2 trypanosomes/mL.

To prepare the samples for analysis, a modified version of the Chelex method was used. To remove residual EtOH from the preserved tsetse tissue, midguts were washed three times as previously described. To each washed midgut, 50µl of a trypanosome DNA concentration was added. Following the addition of trypanosome DNA, 50µl of a Chelex suspension (10% Chelex with a 2% proteinase K concentration) was added to the midgut tissue for a final volume of 100µl. The DNA concentration series was equivalent to $1x10^5$/ml, $1x10^4$/ml,

$1x10^3$/ml, $1x10^2$/ml, $1x10^1$/ml, 1/ml, 0.1/ml, 0.01/ml trypanosomes suspended in 5% Chelex suspension/1% proteinase K. Each DNA dilution was processed according to the standard Chelex extraction method. Table 4.4 summarises the number of trypanosomes in the DNA dilution series per ml, per 100µl, and per reaction for both the LAMP (2.5µl of template) and PCR (2µl of template) assays.

Table 4-4 Dilution gradient of trypanosomes expressed as trypanosomes/mL and as ng/µL.

		Dilution gradient trypanosomes per mL:							
		1x10^5	**1x10^4**	**1x10^3**	**1x10^2**	**1x10^1**	**1**	**0.1**	**0.01**
Trypanosomes/mL	**Trypanosomes/ml**	100000	10000	1000	100	10	1	0.1	0.01
	per 100µl sample	10000	1000	100	10	1	0.1	0.01	0.001
	per LAMP assay	250	25	2.5	0.25	0.025	0.0025	0.00025	0.000025
DNA ng/µL	**ng/µl LAMP assay**	$2.5x10^{-2}$	$2.5x10^{-3}$	$2.5x10^{-4}$	$2.5x10^{-5}$	$2.5x10^{-6}$	$2.5x10^{-7}$	$2.5x10^{-8}$	$2.5x10^{-9}$
	per PCR assay	200	20	2	0.2	0.02	0.002	0.0002	0.00002
	ng/µl TBR assay	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$	$2x10^{-6}$	$2x10^{-7}$	$2x10^{-8}$	$2x10^{-9}$

4.2.6 Pooling

Infected *G. m. morsitans* flies were generated by adding 200µl of thawed *T.b. brucei* GFP J10 blood stabilates to five mL of defibrinated horse blood (TCS Biosciences Ltd). Teneral flies were fed through a silicon membrane placed over the blood which was heated to 37˚C. After seven days the flies were dissected and their midguts visually screened for infection using a compound microscope. Positive midguts were then stored individually in 100% EtOH and later they were processed using the chosen simplified DNA extraction method. Parallel to the infected flies a group of uninfected flies were also generated using the same method as the positive flies with the omission of 200µl of *T. b. brucei* blood stabilate being added to 5 ml of horse blood. The midguts of negative flies were screened prior to being stored in the EtOH with microscopy and later, after DNA extraction using the Chelex method, with TBR PCR to ensure they were truly uninfected.

Pools of tsetse flies were generated by adding 3µl of DNA template from a single positive fly sample to pools of four, nine and 19 uninfected fly samples, in which each fly contributed

3µl of DNA extract to the pool. A total of 9 replicates were performed per pool and each pool was tested with LAMP.

4.2.7 Persistence of DNA in tsetse material

To assess the ability of the Loopamp^{TM} kit to detect trypanosomes from a previous blood meal, 200µl of a 10^6 trypanosome/mL *T. b. brucei* blood stabilate was incubated for 15 minutes at 54˚C to kill the trypanosomes and hence prevent establishment of an infection in the tsetse. The heat-killed trypanosomes were added to 5ml of defibrinated horse blood. The viability of the parasites was determined through microscopy and the use of the stain Trypan blue(372). If no living trypanosomes were observed, the blood was then fed to teneral flies. A similar volume of uninfected blood was heated and fed to a control group of flies. At 24 h intervals after the initial blood meal, three experimental flies and one control fly were dissected and the midgut removed and stored in 95% EtOH. During each dissection the midgut samples were screened visually for living trypanosomes using a compound microscope, at 250x magnification, as a secondary measure to ensure no trypanosomes had survived and had started to infect the flies.

The midgut samples were processed using the Chelex method followed by LAMP and PCR assays as described. The experiments were repeated until the LAMP and PCR tests showed two consecutive days of negative results in all experimental fly samples.

4.2.8 Mixed infections

LAMP kits were tested for cross reactivity with single and mixed species infections of both *T. congolense* (1/148) and *T.b. brucei* (GFP J10). Flies were infected by artificial membrane feeding (373). Partially fed flies were removed. On the seventh day post-infection all flies were dissected and their midguts screened for trypanosomes by microscopy.

The four groups were processed using the Chelex method and analysed using the *Trypanozoon* specific TBR primers and the universal ITS primers alongside the RIME LAMP kits.

4.2.9 Wild-caught tsetse flies

From NW Uganda, 449 *G.f. fuscipes* were caught using pyramidal traps and screened by microscopy, universal ITS primers and the LAMP kits. The flies were caught from April to June 2013 from eight trap sites along the Kochi River (Northings 381161-383674, Eastings 276506-287545) in the district of Koboko. The flies were dissected in the field and their mouthparts, salivary glands and midguts were separately screened for trypanosomes by microscopy. All samples were then stored individually in 100% EtOH and shipped at room temperature to LSTM where they underwent the Chelex extraction prior to PCR and LAMP analysis.

4.3 Results

4.3.1 Simplification of DNA extraction

The results for the six different DNA extraction methods, tested with the LAMP kits, are shown in Table 4.5. The method that produced the highest number of positive results was '½ time Chelex 'followed by 'TE with Chelex' detecting 13 and 12 positive samples respectively out of a total of 16 positives. The lowest scoring methods were 'TE Leave in alcohol' and 'TE wash off alcohol'. Taking into account the number of steps involved for each method and the total time it takes 'TE with Chelex' was deemed the most efficient as it took half the time of '½ Chelex' but only identified one less positive sample (Table 4.5 and Figure 4.2).

Table 4-5 Overview of the results for the different DNA extraction methods.

DNA extraction method	LAMP positives per Trypanosome concentration				Total number of LAMP positives	% of positive samples identified
	10^2 Midgut	10^2 Abdomen	10^4 Midgut	10^4 Abdomen		
TE Leave in alcohol	2	0	3	2	7	44
TE wash off alcohol	1	0	4	4	9	56
½ time Chelex	4	2	4	3	13	81
Chelex	3	1	4	3	11	69
TE with Chelex	4	4	3	1	12	75
¼ Chelex	3	2	3	3	11	69

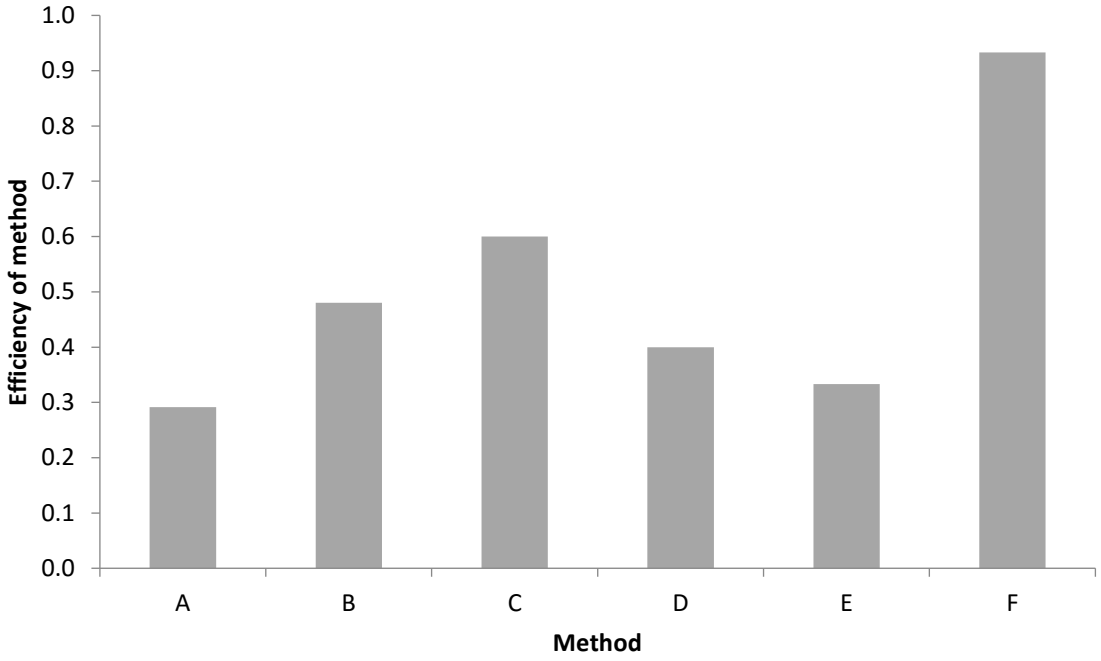


Figure 4.3 Results of the efficiency formula for different DNA extraction methods:
A= Chelex, B= ½ time Chelex, C= ¼ Chelex, D= TE leave in alcohol, E= TE wash off alcohol and F= TE with Chelex

4.3.2 Limit of Detection

The results (Figure 4.3) show that the LAMP kit was able to detect 100% of the spiked samples up until 10^4 trypanosomes per ml, after which there was a decline in the ability to detect the trypanosome DNA until 10^{-1} trypanosomes per ml. The sensitivity of the LAMP kit when compared with the TBR PCR, at the extreme limit of detection, was better by a factor of 100 as the TBR PCR had a limit of detection of 10^1 trypanosomes/ml. Although the template volumes for the LAMP and TBR assays varied slightly by 0.5 µl (or 25%) this variation is too small to explain the two fold, (or 100 x) greater difference in the sensitivity of the two assays.

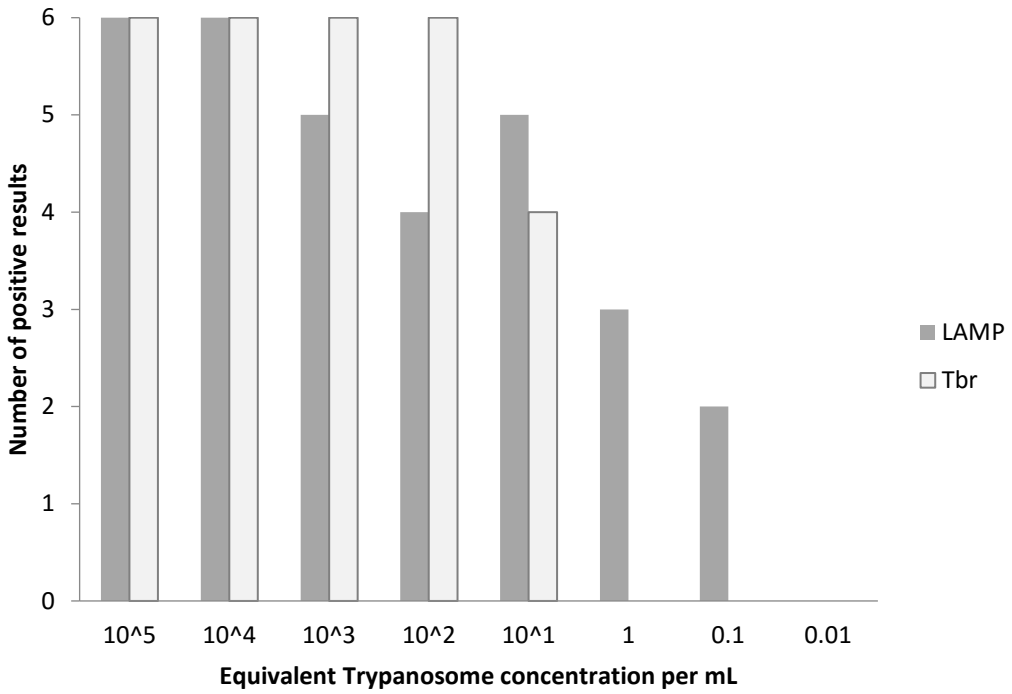


Figure 4.4 Limit of detection results for LAMP and TBR primers:
a total of six flies were used for each dilution gradient for both assays.

4.3.3 Pooling

Pooling experiments show that LAMP was able to amplify one positive midgut when pooled with four, nine and 19 uninfected midguts - this was repeated in nine independent tests. There were two kit failures, one in ratio 1:5 and the other in 1:20. The failure was caused by the dried reagents failing to properly dissolve and integrate into the reaction.

4.3.4 DNA persistence

The number of tsetse that were able to give a positive LAMP results declined from 100% after 48 hours to just 11% by day six and zero by day seven(Fig 4.4).

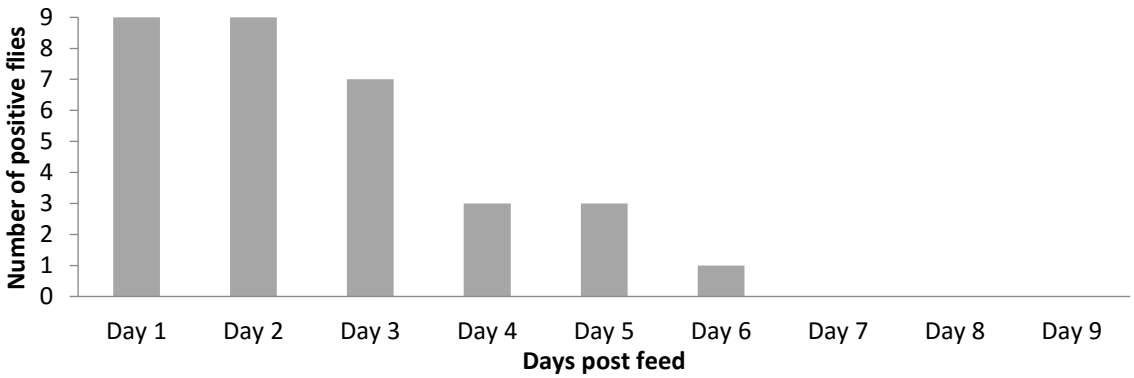


Figure 4.5 *T. b. brucei* DNA time series
Tsetse were fed dead trypanosomes on day 1 and were sampled every 24 hrs, producing a time series of DNA persistance. The flies were fed every 48 hrs with a fresh blood meal.

4.3.5 Mixed infections

Thirteen out of sixteen surviving flies from single *T.b. brucei* infection were identified as positive by both microscopy and TBR PCR (Table 4.6). When the same samples were tested with LAMP, all 16 flies tested positive. Of the 17 surviving flies from the *T. congolense* single infection, 14 were positive by microscopy, 13 tested positive with the ITS primers, which amplifies and differentiates both *T. brucei and T. congolense,* and none were detected by LAMP, which is specific for *T. brucei s.l.*. Of the mixed infection group both LAMP and Tbr did not cross react with *T. congolense* and the present of non-target DNA did not inhibit the amplification of *T. b. brucei* DNA.

***Table 4-6 Results from single and mixed infections of T.b. brucei and T. congolense*.**
Groups of 25 tsetse were infected with *T. b. brucei* and *T. congolense* as mono and mixed infections. These groups were screened with microscopy, PCR and LAMP. A control group of 25 tsetse was included.

Infection	Assay	Positive	Negative	Lost Flies	Total
Single *T. brucei* infection	LAMP	16	0	9	25
	TBR	13	3	9	25
	Microscopy	13	3	9	25
Single *T. congolense* infection	LAMP	0	17	8	25
	ITS	13	4	8	25
	Microscopy	14	3	8	25
Mixed infection *T. b. brucei* and *T. congolense*	LAMP	20	3	2	25
	TBR	17	6	2	25
	ITS	18	5	2	25
	Microscopy	15	8	2	25
Negative control	LAMP	0	16	9	25
	TBR	0	16	9	25
	ITS	0	16	9	25
	Microscopy	0	16	9	25

4.3.6 Wild flies

Of the 449 wild-caught tsetse, microscopic examination of tsetse identified one salivary gland positive *T. brucei s.l.* sample, the universal ITS primers identified two *T. brucei* sl. samples, including the microscopy positive, while LAMP identified six positives, including the microscopy positive tsetse but only one of the two samples identified by the universal ITS primers. The ITS assay identified 40 flies positive for non-*Trypanozoon* trypanosomes (*T. vivax* n:11, *T. congolense s.l.* n:5, *T. grayi* n: 22, *T. simiae* n:2) which the LAMP kits did not cross-react with.

4.4 Discussion

4.4.1 Overview

This study has demonstrated the potential of the Loopamp *Trypanosoma brucei* detection kit as a tool for identifying *Trypanozoon* DNA in tsetse. The LAMP kits showed a high degree of specificity, with no cross-reaction when non-target species of trypanosome are also preent. LAMP was more sensitive than standard PCR in both laboratory-infected and wild-caught tsetse flies. Further it was shown that a single trypanosome-infected tsetse could be detected in a pool of 20 flies. Simplified DNA extractions were developed and the results show the ability to reduce the time of DNA extraction, although moderate equipment and skill is still required. The results suggest that LAMP may be a useful tool for epidemiological surveillance of *T. brucei s.l.* in HAT endemic regions to estimate the burden of the disease (in combination with species-specific PCR). This may be more cost-effective than active detection of human cases, and would allow control programmes to prioritise areas for HAT control.

4.4.1 Simplification of DNA extraction and Pooling

The field extraction methods tested here have been assessed by balancing the length of the process, reliability at detecting positive samples and the number of steps involved in the method, which is a proxy for its complexity. The most successful method at identifying spiked samples was the '½ time Chelex method' but once the complexity and time was taken into account 'TE with Chelex' was decided as the most efficient DNA extraction method. 'TE with Chelex' was also able to detect all the samples, both midgut and abdomens spiked with the lowest concentration of trypanosomes, although it was unable to do the same with the higher concentration of trypanosomes. This was especially true with the abdomens spiked with 10^4 trypansomes, where only 1 out of the four samples processed with this method was LAMP positive. This discrepancy could be due to inhibitors in the tsetse midguts or surrounding tissue. However, all of these extractions still require access to centrifuges, multiple reagents and handling steps and must be improved before implementation.

In previous studies, LAMP assays on pooled samples showed a decline in sensitivity by 60% with a ratio of 1:15 (154). In the present study, the LAMP kit was capable of detecting one

infected tsetse amongst 19 uninfected flies 100% of the time when coupled with the TE Chelex extraction method. This demonstrates that both the kit and the TE Chelex extraction have good potential for use as tools in the xenomonitoring of tsetse.

4.4.2 Limit of Detection

The serial dilution experiment confirms that the LAMP kits have an extremely high sensitivity in detecting trypanosomes. Previous studies have reported limits of detection ranging from the equivalent of 100 to 0.001 trypanosomes per ml (152, 154, 364) using in-house LAMP assays in accordance with results shown here. The advantage of using a commercially available, standardised LAMP kit would allow researchers to compare different settings with each other without differences in method confounding the results. The ability of the LAMP kits to detect such a low number of trypanosomes per ml is advantageous for its use in xenomonitoring as flies actively transmitting the disease can have infections ranging from $<1 \times 10^1$ to 1×10^4 depending on the stage of infection(374). However, when viewing the results of the TBR and LAMP it is clear that the reliability of the two assays differ as although LAMP can detect lower concentrations of trypanosomes it also has a higher rate of false negatives at detectable levels of trypanosomes DNA, as is shown in Fig 4.3 where the Tbr PCR was capable of detecting six out of the six samples at 10^3 and 10^2 trypanosomes per mL however the LAMP assay was only positive for five and four of the six samples respectively. This may be due to inhibitors in the DNA template and the nature of how the results are viewed post-reaction. The TBR results need to be run out on a gel and the gel is then read using a gel imager. By using the gel imager even very faint bands in the gel can be detected by adjusting exposure times, contrast and brightness levels whereas the LAMP results are viewed solely by the naked eye.

4.4.3 DNA Persistence

Another, less obvious, advantage with xenomonitoring is that the tsetse flies are continuously feeding on a wide range of local vertebrates (290) and could, in effect, act as an efficient source of blood for screening in order to detect *Trypanozoon* species in the environment. If tsetse are able to pick up *T. brucei s.l.* trypanosomes in blood meals and trypanosome DNA can be amplified, it would be possible to detect if HAT is present in a

region. The results for *T. b. brucei* DNA persistence are highly encouraging as they demonstrate that it is possible to detect DNA reliably from a blood meal for 48 h post-feeding. This is despite the fact that the tsetse flies in the present study were not only digesting the initial infected blood meal, but they subsequently took on fresh blood, at later feeds, which helped flush their digestive system of previous meals.

4.4.4 Mixed Infections

The situation in the field may also be complicated by the presence of other trypanosome co-infections in the tsetse flies (254). The results from the co-infection experiments demonstrate that even with co-infected flies it was still possible to identify those that had a *T.b. brucei* infection and in the one case where the fly cleared the *T.b. brucei* infection but maintained a *T. congolense* infection, the LAMP kit recorded this as a *T.b. brucei* negative fly, Table 4.6. The specificity of the LAMP kits was good when tested against single infections of *T. congolense* and *T.b. brucei*, with no cross-reaction with the flies infected with *T. congolense*. In wild-caught flies, the LAMP test did not cross react with the wild populations of *T. vivax, T. congolense, T. grayi* and *T. simiae*. The LAMP kits only amplified one of the two *T. brucei s.l.* identified by the universal ITS primers. This could be due to inhibitors in the sample, although this is unlikely as the LAMP kits are capable of oricessing whole blood (375). An alternative explanation would be that an unknown strain or species of trypanosome was amplified from the tsetse producing a similar sized band to *T. brucei* s.l..

The LAMP kit used in the present study is only able to identify the *Trypanozoon* group*,* meaning that a species-specific PCR would still be necessary to differentiate human infective forms from the other trypanosomes in order to guide HAT control programmes effectively. Unfortunately, the species-specific PCRs used to differentiate the *Trypanozoon* group use single-copy gene targets (376, 377), making them significantly less sensitive than LAMP and other PCRs (e.g., TBR, ITS). In addition, it is currently not possible to conduct LAMP in a high-throughput manner, and the cost of kits is still significant, at $2.60 per test. A larger study involving cost analysis of PCR vs LAMP should be conducted to verify that molecular xenomonitoring is a suitable tool for studying the epidemiology of HAT and is able to highlight hotspot areas of transmission.

4.4.5 Conclusion

This study has demonstrated a robust detection limit of the DNA dilution equivalent to 1 trypanosome/10mL for the *Trypanozoon* detection kit. The kit has been shown to have a high specificity, with no cross-reactivity in flies with multiple infections, including wild caught flies that had a greater variation of non-target species and provided a more realistic challenge. The kits could also detect *T.b. brucei* DNA 6 days after consumption of a contaminated blood meal, despite having fresh feeds every 48 hrs, which would be flushing their digestive system with clean blood. The sensitivity of the kits was high enough to allow for the detection of a single infected fly in a pool of 20 flies. The next step would be to test the DNA extraction and pooling methods in the field alongside a wider cost and feasibility evaluation of LAMP vs PCR and the ability to use xenomonitoring of HAT over time in the elimination campaign.

Chapter 5 : Impact of tiny target intervention on zoonotic trypanosome transmission

5.1 Introduction

The control of West African sleeping sickness has historically been reliant on case detection and treatment ever since it was successfully employed by Eugène Jamot in 1916-1917. Jamot termed his intervention “medical prophylaxis” and in the 18 months of this intervention close to 90,000 people were examined and over 5,000 people, identified as gHAT positive, were treated with atoxyl (378, 379). Active screening and treatment of populations at risk of gHAT by mobile teams however this has since been scaled back in the decades following de-colonisation (380) but has since been renewed in the 21st century (381). The role of vector control as a means of combating sleeping sickness is mainly a feature of combating East African sleeping sickness and has been used far less frequently in West Africa. The high number of targets needed to control the vectors of West African sleeping sickness has for a long time made this prohibitively costly.

This hurdle of cost effective West African tsetse control methods has now been overcome after experimentation with target sizes revealed that a 90% reduction in target area led to a 50% reduction in catch numbers (231). It was found that the number of tsetse caught per m^2 of fabric is optimum with 25 cm^2, targets flanked by an equal area of netting (231). Due to their smaller size the reduction in the cost of the fabric offsets the number of targets required for effective control resulting in an economically viable strategy (234, 235). This increase in tsetse killed per m^2 of fabric means that the cost of controlling tsetse with the new, smaller targets, is USD 55.7 per km^2 (382) which is far below previous estimated costs for tsetse control which ranged from USD 179 to USD 556 (383, 384).

This new, smaller, target technology has been dubbed “tiny targets” and with this new approach to vector control a major shortfall of case detection and treatment can be overcome, namely that the screening of the human population is never complete (385). Studies have shown that active screening coverage can vary from as low as 10% (386) and rarely exceeds 70% (387, 388) and it does not have an immediate effect on reducing the risk

of being infected via the bite of tsetse (236). The lack of 100% coverage is due to multiple factors from hard to reach communities to taboos associated with the screening process that involve fear of treatment, fear of being diagnosed as gHAT positive and the subsequent loss of work and a distrust of the mobile screening teams (387). Initial studies, conducted on islands on Lake Victoria, showed that the optimum number of Tiny Targets to be deployed was 20 per linear km (236). The Tiny Targets reduced the tsetse population by >90% and this result was replicated when the Tiny Targets were deployed on a larger scale in N W Uganda in 2013 (236).

5.1.1 Tiny targets intervention NW Uganda

Full details of the Tiny Targets trial in NW Uganda are described by Tirados *et al* 2015 and summarised here. The Ugandan study was carried out in two phases and lasted from September 2011 to December 2013. In the first phase, September 2011-December2012, the Tiny Targets were deployed in five separate 7x7km blocks, in the historical HAT foci of Arua and Maracha, producing a total discontinuous intervention area of 250km^2 (Fig. 5.1.A). Later, in the second phase from January 2013 to December 2013, the study site was increased in size to 500km^2 by deploying Tiny Targets between the five original intervention blocks to link them up into one large intervention zone (Fig. 5.1.B). The targets were deployed within the following co-ordinates: 3.053°-3.310° N and 30.838°-31.082° E.
The impact of the Tiny Targets on the tsetse population was assessed using pyramidal traps located in the centre and edges of the five original blocks; an additional 12 more traps were added in phase two when the intervention site was expanded. To the North of the study site a similar ecological environment was selected in the HAT foci of Koboko, along the Kochi River, from 3.451°-3.465° N and 30.058°-31.003° E. This site was selected as an experimental control site and no targets were deployed within this region. Within this non-intervention zone fifteen monitoring sites were selected to assess the fly prevalence throughout the course of the study.

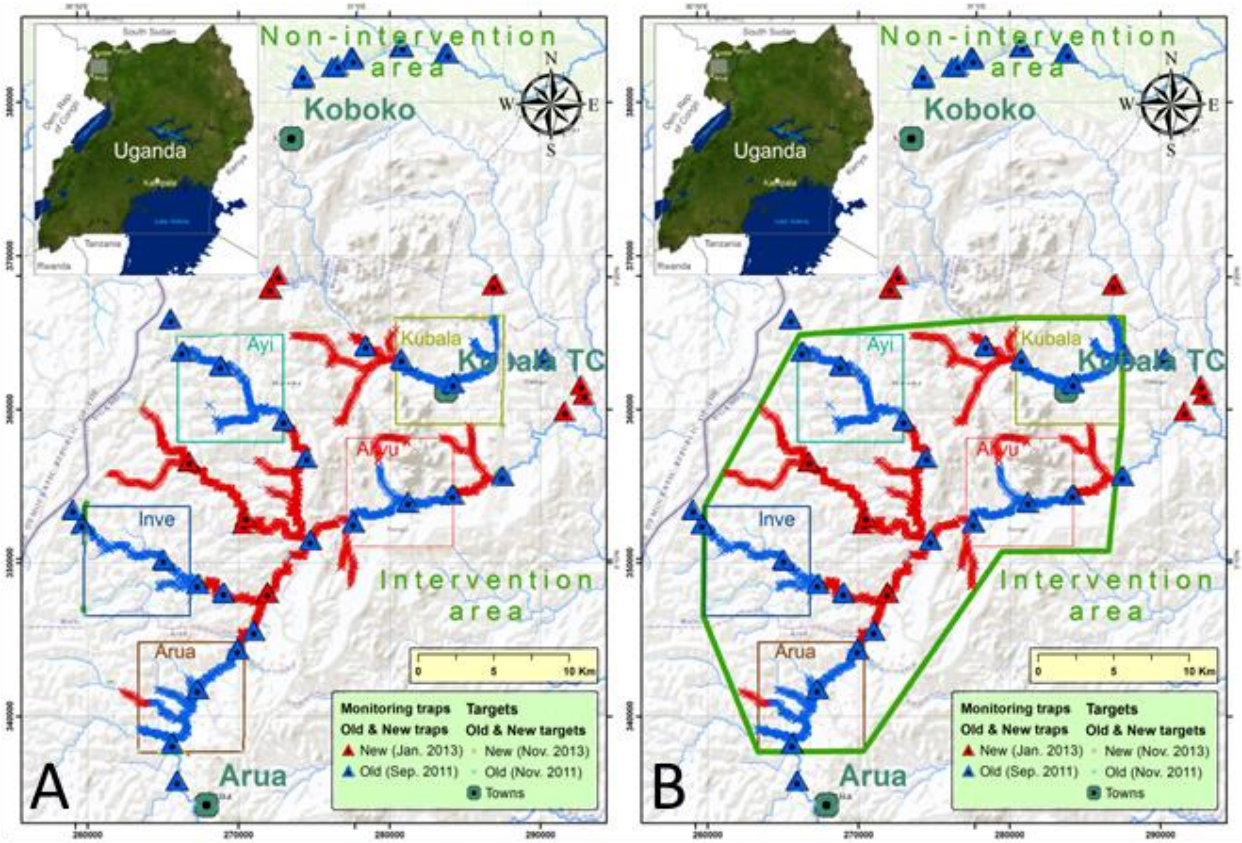


Figure 5.1 ***Tiny Target deployment sites***
The initial 7x7 km intervention blocks (A) were Ayi, Kubala, Inve, Aiyu and Arua. They were separated from each other by areas lacking tiny targets. These five sites were later linked up as new tiny targets were distributed between the intervention blocks, expanding the treatment area to a 500km^2 site (B). Image provided by Tirados (389)

The entomological survey in both sites showed that the Tiny Targets had a significant impact on the tsetse numbers caught in the intervention zone whereas the tsetse population from Koboko showed little change across the intervention period (236). Although the impact of the intervention on the numbers of tsetse in the area is clear the effect on the transmission of salivarian trypanosomes has not been determined.

The previous studies evaluating the effectiveness of the tiny target technology have concentrated on the effects the targets have on tsetse numbers. This is the first study to attempt to evaluate the effects of the Tiny Targets on the transmission of trypanosomes of medical and veterinary importance. In order to evaluate the impact the Tiny Targets have on the transmission of salivarian trypanosomes cattle from both the intervention and non-intervention site will be screened for *T. brucei s.l., T. congolense* and *T. vivax*. Of primary interest is the effect of the intervention on the transmission of *T. brucei s.l.* as this species complex includes the pathogen responsible for gHAT. Any observable reduction in this

species complex across the intervention site will support the argument for this technology to be used to combat gHAT. The other two common salivarian trypanosomes, *T. congolense* and *T. vivax* will also be screened for due to the relative low prevalence of the *T. brucei s.l.* infections and help increase the chance of detecting a significant difference in transmission of trypanosomes between the two sites.

5.1.2 Animal reservoirs of HAT

The zoonotic nature of Rhodesian sleeping sickness has been proven for some time (390) and Gambian African sleeping sickness is widely accepted to be an anthroponotic disease. However, there is still a question over the importance of an animal reservoir host within the transmission cycle of *T. b. gambiense*. Studies have found domestic animals positive for *T. b. gambiense* in pigs, sheep and goats (64, 391) as well as numerous non-domestic animals (63). However, despite finding *T. b. gambiense* in non-human hosts there is no evidence that these animals are actively part of the transmission cycle. The elimination of *T. b. gambiense* from the island of Bioko through screening and treatment of the human population (392) would suggest that there was no animal reservoir. Similarly, the success of the case detection and treatment approach in reducing the number of West African cases of sleeping sickness across large parts of the continent would also support the theory that this is an anthroponotic disease without an animal reservoir. Other studies seeking to find *T.b. gambiense* amongst domestic animals from historic HAT foci have failed to find any domestic animals positive for the gambiense sub-species (393). However, the potential of an animal reservoir cannot be overlooked if the push to elimination is to be achieved by 2020. Recent mathematical models have shown that the ability to eliminate the disease can be heavily influenced by non-human reservoirs of *T. b. gambiense* (394). The old transmission site at Bioko which once supported the notion of an anthroponotic disease has recently led credence to an animal reservoir argument as the parasite is still being found in the local tsetse population despite no evidence of it being in the human population. This suggests that there may be an animal reservoir maintaining transmission (60).

5.1.2.1 Cattle and pigs as potential reservoirs for T. b. gambiense in NW Uganda

Having identified *T. brucei s.l.* in the local cattle populations from the HAT focus of Arua and Maracha, as presented and discussed in chapter 5, these Tbr-FIND positive samples would need to be identified down to the species to determine whether or not *T. b. gambiense* was present in any of the cattle. To complement the study of cattle, pigs were also sampled for trypanosomes since they have previously been implicated as a potential reservoir host for *T. b. gambiense* (64, 65, 395-397) and since the mid 1980's pig production has become an important income-generating activity in Uganda (398). A study carried out from 2004-2008 screened domestic animals in NW Uganda and found that pigs had the highest percentage positive rates (21.7%) for *T. brucei s.l.* using the Tbr primers. This was despite only 161 pigs being screened out of a total of 3,267 other domestic animals. Cattle, goats and sheep all had a lower Tbr positive rate at 14.5%, 12.4% and 10.8% respectively (393). Although this study failed to find any animals positive for *T. b. gambiense* it is unclear precisely where the samples were collected. In this study, samples were collected close to areas where HAT cases had been recently reported. The results of the 2012 study are also unreliable as the Tbr positive samples were not tested with a single-copy *Trypanozoon* primer set, meaning that the absence of products from the TgsGP primers could be due to insufficient DNA present for the amplification of a single copy gene as opposed to a true negative result (271).

5.1.3 Trypanosoma theileri

Aside from the salivarian trypanosomes there are a number of non-tsetse transmitted species chief among these is *Trypanosoma (Megatrypanum) theileri*. This is a large trypanosome (Fig.5.2) that is found throughout the world's cattle (128, 131, 249, 399). The vectors of *T. theileri* are tabanids (400) and generally cattle infected with this parasite show little to no clinical symptoms (400, 401). *T. theileri* infections can be significant amongst cattle populations reaching levels as high as 81-86% (249, 402).

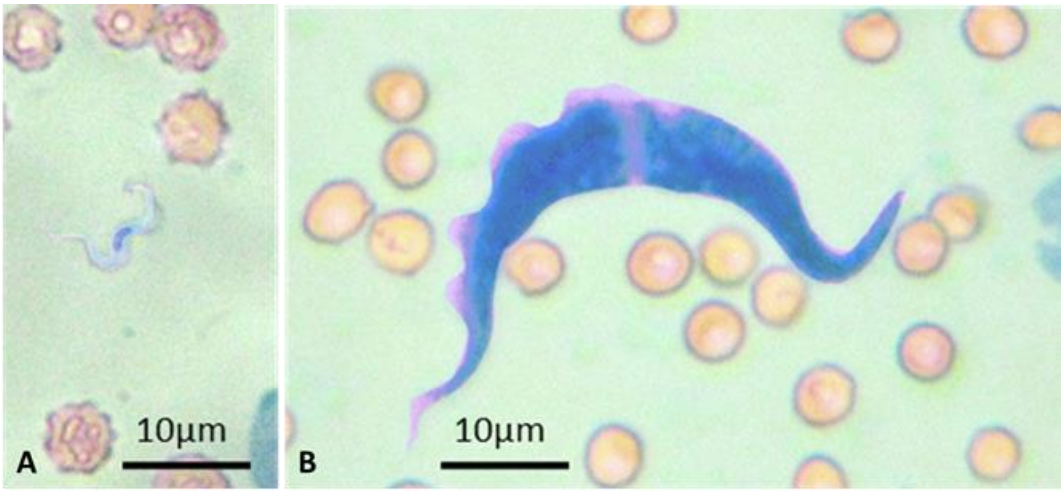


Figure 5.2 ***Size difference between T. theileri and salivarian trypanosomes***
Comparison in size between an unknown salivarian trypanosome species (A) and Trypanosoma (Megatypanum) theileri *(B) isolated from Ugandan cattle*. The samples were stained with giemsa. Trypanosome A is most likely to be either *T. vivax* or *T. brucei s.l.* based on its size in relation to the red blood cells. Images taken during this study.

The presence of the non-salivarian trypanosome *Trypanosoma theileri* may confuse the interpretation of both microscopy results and molecular analysis especially the ITS results due to its high genetic variability (273, 403) which results in a range of PCR product sizes.

5.1.4 Objectives

As mentioned previously the impact of Tiny Targets on the numbers of the local vector population has been well studied (236) however the impact of this novel vector control strategy on the transmission of salivarian trypanosomes and the health of local host animals has not been investigated. Therefore, the purpose of this chapter is to address these gaps in the current knowledge of the impact of the Tiny Targets in the wider context of trypanosomiasis transmission and morbidity.

Aims:

1. The prevalence of trypanosomiasis in cattle populations from the intervention and non-intervention sites will be quantified to determine the impact of vector control.
2. Assess the presence of *T. b. gambiense* in local cattle and pig populations by:
    i. Determine the prevalence of *T. brucei s.l.* in the local pig populations from two sites in NW Uganda
    ii. Establish how many *T. brucei s.l.*, cattle and pigs have enough genomic material to detect a single copy gene
    iii. Using the *T. b. gambiense* specific primers assess whether individual cattle and pigs that are *T. brucei* s.l. positive were infected with T. b. gambiense

5.2 Methods

5.2.1 Cattle sampling

To determine the effects of the intervention on the transmission of salivarian trypanosomes in the local cattle population a baseline for trypanosome prevalence was established by screening a total of 406 animals from the following five 7x7km intervention blocks: Aiivu (80), Arua (74), Ayi (70), Inve (65) and Kubala (117), a further 200 cattle were screened from the non-intervention zone in Koboko from two sites hereafter called Koboko East (100) and Koboko West (100). The selected cattle were treated with Albafas 10% de-wormer, main active ingredient albendazole, to encourage the co-operation of the local farmers, and the trypanocides Berenil or Veriben, main active ingredient is diminazene aceture. The de-wormer was administered at 750mg of albendazole per 100kg of animal body weight and the trypanocide was administered at 350mg of diminazene aceture per 100kg of animal body weight.. To track the treated cattle, they were tagged with numbered ear tags of differing colours. This initial, pre-intervention, sampling will be referred to as round 0 (R0) and was used to inform the power calculations for the seven sampling rounds that followed (R1-R7)(Table 5.1).

Table 5-1 Time frame of the sampling rounds and corresponding control operation.

Sampling Round		R0*	R1	R2	R3	R4	R5	R6	R7
Dates	**Start**	01/08/11	15/12/11	30/04/12	01/08/12	17/01/13	02/05/13	20/08/13	26/11/13
	End	20/08/11	25/01/12	11/05/12	10/08/12	26/01/13	14/05/13	28/08/13	04/12/13
Control operation			1st Phase			2nd Phase			

*R0 was carried out prior to the intervention.

5.2.7.1 Power calculation

Prior to carrying out the study the appropriate sample size of cattle for the intervention and non-intervention sites needed to be calculated in order to be sure that a large enough sample size has been used for the interests of the study. In this case our primary interest is to detect changes in the prevalence of trypanosomiasis in the cattle populations from the areas in which Tiny Targets were deployed and the areas in which no targets were present. In theory a decrease in the vector population would impact the transmission of disease.
To calculate the required sample sizes the following power calculation was used (404):

$$n = \left(t \frac{\sqrt{P}(1-P)}{l} \right)^2$$

Where: n= sample size, t= students T value, taken to be 1.96 for a 95% confidence level, P= prevalence, taken to be 15% from screening results, l= absolute level of power at 5%.

These parameters were input into the equation to give:

$$n = \left(1.96\frac{\sqrt{0.15}(1-0.15)}{0.05}\right)^2 = 196$$

During each round, the same sampling sites were revisited and cattle that were treated in the previous round were re-sampled. If there was a short fall in the number of cattle, below 196, new animals were recruited to the study. The trypanocide, veriben, was used as it remains effective for ~10 days and does not provide long-lasting protection, allowing the cattle to become re-infected. A reduction of tsetse numbers due to the vector control is expected to be reflected in the proportion of cattle that become re-infected. The cattle sampling lasted from August 2011 to November 2013, and on average each sampling round lasted 16 days with 105 days separating the end of one sampling round and the start of the next. The cattle sampling methodology is depicted in a diagrammatic form below (Fig.5.3.).

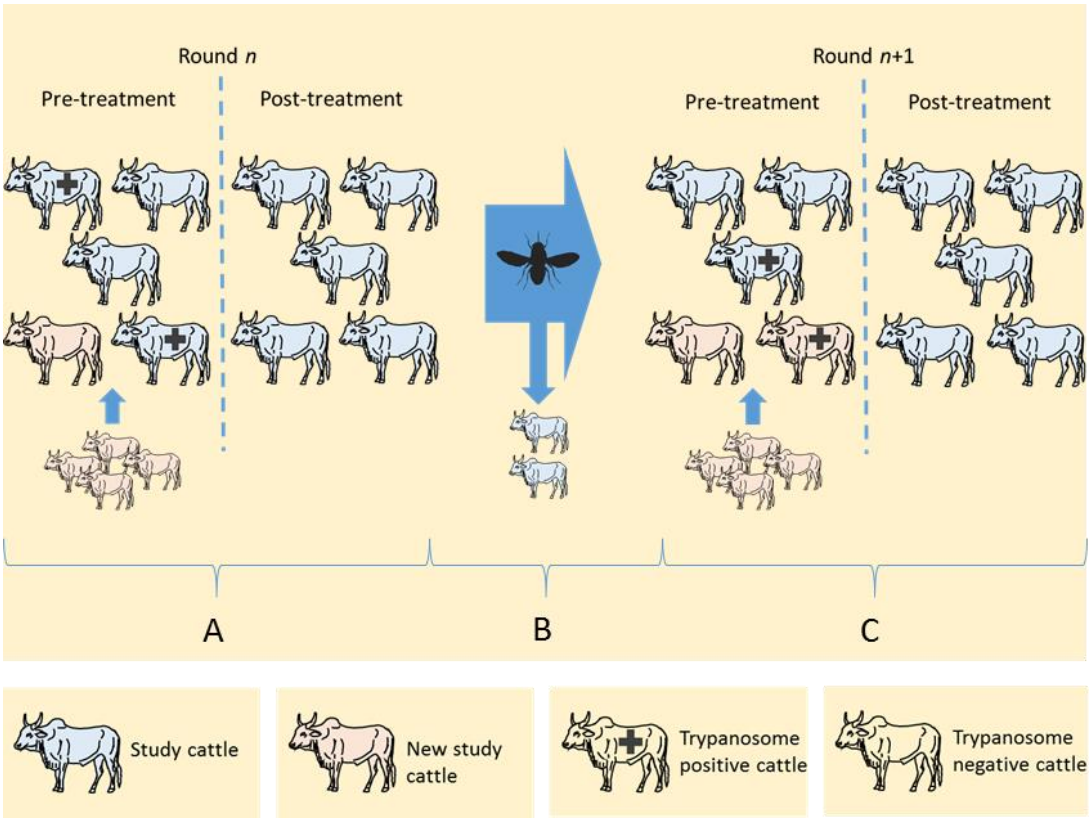


Figure 5.3 ***Sampling method employed covering two consecutive rounds:***
in the initial round (A), cattle are being re-sampled and any loss in numbers are replenished with new cattle to reach 196. Blood samples are then taken and the cattle treated with veriben to clear them of trypanosomes. (B) Between the two sampling rounds cattle are lost and are susceptible to re-infection. (C) The cattle from the previous sampling round are then re-sampled and new cattle recruited to replace those lost.

5.2.2 Pig sampling

The NW of Uganda has seven districts, Nebbi, Arua, Koboko, Yumbe, Moyo, Adjumani and Gulu, of which Arua, Koboko, Yumbe, Moyo and Adjumani have historic sleeping sickness foci (405). This study includes cattle data from Arua and Koboko and pig data from Arua (Fig.6.1.A) and Moyo (Fig.5.1.b). It was not feasible to sample pigs from Koboko as this is a predominantly Muslim area where there are very few pigs.

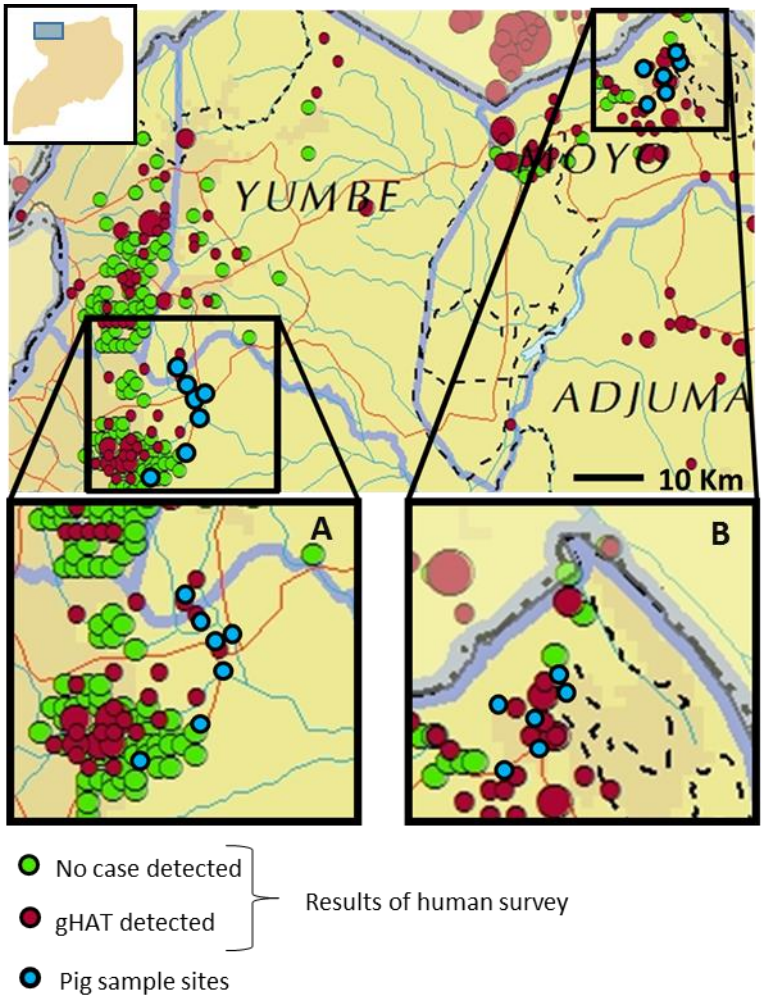


Figure 5.4 Distribution of the pig sampling sites:
(A) Arua and (B) Moyo overlaid on the gHAT distribution sites from 2010-2014. Image adapted from the Atlas of HAT (406).

5.2.3 Blood collection

Blood was collected from individual cattle by first piercing a vein in the ear with a disposable lancet and collecting three, 35µl, samples of blood, using 50mm heparinised capillary tubes. One of these samples was directly spotted onto FTA card and left to air dry out of direct sunlight. The other two blood samples were then further processed to establish the PCV values and microscopy positive status as described below.

5.2.4 Microscopy

Following on from the PCV reading a wet slide was then prepared from the buffy coat layer of one of the tubes as initial screen for trypanosomes using a Leica DM500 microscope equipped with a dark field filter at 200x magnification. Using this method, it was not

possible to differentiate the species of trypanosome, so all positives were included in this section of the study.

5.2.5 FTA DNA extraction

The FTA cards were used to store individual blood samples from the study animals. The blood spots were left to air dry and then placed in a heat sealed foil pouch with silica beads. FTA cards were then transported back to the UK and processed at the Liverpool School of Tropical Medicine using the methods described in chapter 2 section 2.2.1 and is summarised below.

5.2.6 Molecular analyses

5.2.6.1 T. brucei s.l.

All samples were screened with the *T. brucei s.l.* specific Tbr-FIND primers described in Chapter 2, these primers target a 10,000 copy region of the genome and have a greater sensitivity than the generic ITS primers.

5.2.6.2 ITS

In addition to the *T. brucei s.l.* specific primers generic ITS primers were also used to identify *T. brucei s.l., T. congolense* and *T. vivax*. Identification required running the PCR products out on a 1% agarose gel stained with ethidium bromide and measuring the band sizes.

5.2.6.3 Species specific PCR and sequencing

Putative *T. congolense* and *T. vivax* samples were re-tested with species specific primers described in Chapter 2 for the three strains of T. congolense and West African strains of T. vivax. 18S sequencing primers were used to help confirm the species and sub-species for samples of interest. The PCR products designated for sequencing were cleaned with the Qiagen PCR clean-up kit and sent for Sanger sequencing to Source-Bioscience.

5.2.6.3 T. brucei s.l. multiplex PCR and T. b. gambiense species specific PCR

As mentioned in Chapter 3 (sections 3.2.5) there is a difference between the sensitivity of the methods used to identify samples positive for *T. brucei* s.l. and those used to identify the

sub-species of *Trypanozoon*. The methods used to identify the sub-species are the same as those used in Chapter 3 and have been previously described in Chapter 2 (section 2.2.5).

5.2.7 Statistics

The impact of Tiny Targets on the infection status of cattle was assessed by comparing the proportions of infected cattle in areas with and without targets. Proportional data are not Normally distributed and so standard statistical tests are inappropriate. Moreover, herds were repeatedly sampled and hence allowance must be made for repeated measures. Accordingly, analyses were carried out using a Generalized Linear Mixed Model (GLMM) with a binomial error distribution. Fixed effects included presence or absence of targets (two levels) and sampling round (seven levels) as fixed effects while the sentinel herd (seven levels) was a random effect. Further details are provided in the Results below.

5.3 Results

5.3.1 Cattle loss and between rounds

The proportion of cattle lost between rounds is shown in Fig.5.4, the most significant drop-out of cattle occurred in the intervention site with a loss of 126 (63%) cattle from R1 to R2. On average the intervention site lost 67 (27%) animals between rounds and the control site lost an average of 54 (29%) animals.

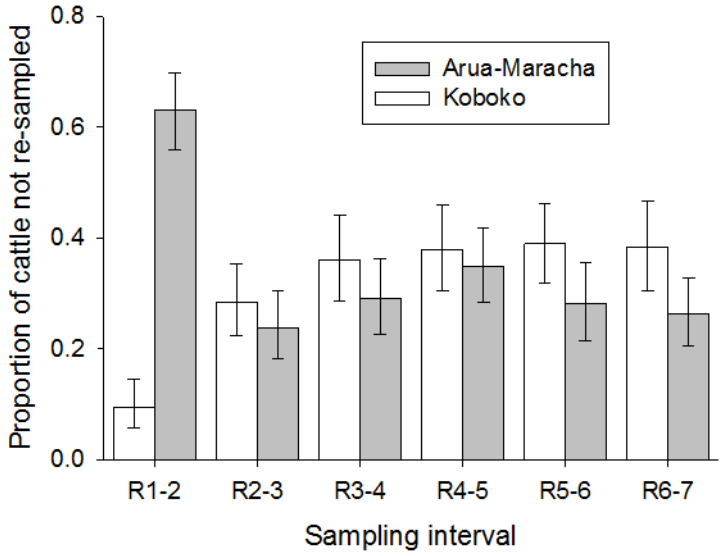


Figure 5.5 Proportion of cattle lost between rounds:
from the intervention (Arua-Maracha) and control (Koboko) sites.

As mentioned in the cattle sampling description the loss of cattle between rounds meant that in order to meet the minimum sample size, new cattle were recruited to the study. New cattle were classified as any animal that had not been sampled in the previous round. This included cattle that had never been sampled and were completely new to the study and cattle that had been sampled in earlier rounds. Table 5.2 summarises the numbers of animals that were re-sampled or added to the sentinel herds.

Table 5-2 ***Numbers of new cattle recruited into the study***

		Round pair					
Sampling site	Sample status	R1 R2	R2 R3	R3 R4	R4 R5	R5 R6	R6 R7
Intervention	re sampled	74	153	129	138	120	157
	Never sampled	127	1	50	5	44	23
	Previously sampled	0	28	33	24	49	17
	Total "new" cattle	127	29	83	29	93	40
Control	re sampled	180	143	99	100	116	90
	Never sampled	20	7	53	52	24	45
	Previously sampled	0	5	9	38	6	20
	Total "new" cattle	20	12	62	90	30	65

5.3.2 Microscopy

The microscopy results were based on screening a wet slide prepared using the buffy coat layer of a centrifuged capillary tube in the field during sample collection. A total of 3173 samples were screened, comprising of 'new' and 'previously treated' animals, 1784 (56%) from the intervention zone and 1389 (44%) from the control zone. Of these, 660 (21%) samples were microscopy positive, 304 (17%) from the intervention zone and 356 (26%) from the control zone (Fig 5.5). Logistic regression analysis of the data showed that there was no significant difference (GLMM, z=-0.155, ns) in the infection rate between herds in areas with (0.16, 95%CI=0.107-0.237) or without (0.21, 95%CI=0.155-0.275) targets.

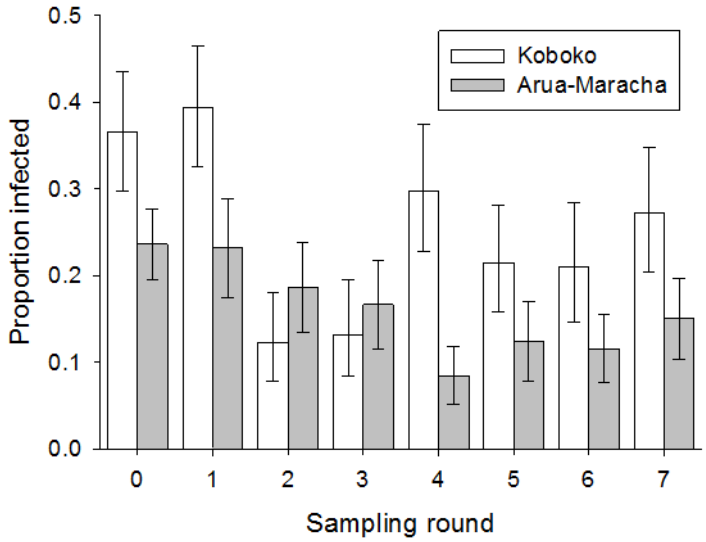


***Figure 5.6 Proportion of cattle with Trypanosoma* spp.**

The results shown in Fig.5.5 show that from Round 4 onwards the intervention site shows consistently lower microscopy positive cattle. However, there was a similar difference before the intervention (Round 0) and these percentages are confounded by new cattle recruited into the study that had not been treated in the previous round.

The results for only those animals present in two consecutive sampling rounds are shown in Fig.5.6. These results show that there was again a reduction in the incidence of *Trypanosoma* infection in the area where targets were deployed (Arua-Maracha). Statistical analysis of the data showed that the difference was however not significant (GLMM, z=-1.49, ns) with the overall proportion infected in areas with and without targets being 0.19 (0.110-0.310, 95%CI) and 0.12 (0.053-0.240) respectively.

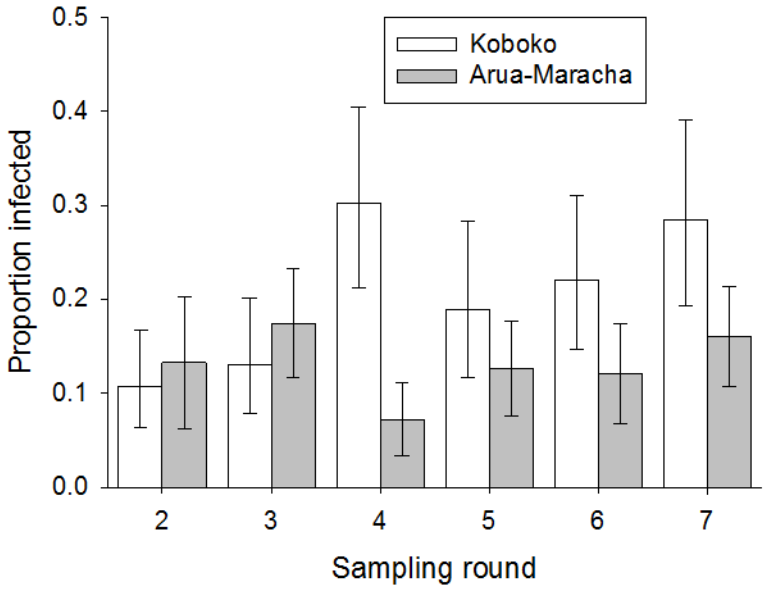


Figure 5.7 Proportion of cattle infected with* Trypanosoma *that were re-sampled
The cattle were sampled three months after being treated with a trypanocide in the previous sampling round. Targets were present in Arua-Maracha only.

The microscopy results show a decrease in the percentage of cattle infected with trypanosomes from round 4 onwards. However, these results include all trypanosomes present in the cattle including *T. theileri* which is not transmitted by tsetse and so would not be affected by the vector control. Hence it is important to consider the salivarian trypanosomes alone. It was not possible to track animals sampled in Round 0 to Round 1 as no records were kept of tagged animals in Round 0.

5.3.3 Tbr positive rate

The primary interest of this study was to see the effects of the vector control on the transmission of *T. brucei s.l.* as within this species-group is the parasite responsible for gHAT. Using the highly specific and sensitive Tbr primers an accurate number of *T. brucei s.l.* infected cattle was produced from each sampling round (Fig.5.7). The total number of *T. brucei s.l.* positive cattle across all sampling rounds was 30 (1.9%) in the intervention site and 8 (0.6%) in the control site. These results show that the majority of *T. brucei s.l.* infections occurred in the intervention area (Arua-Maracha). The prevalence of *T. brucei s.l.* decreased in the intervention site from 4.5% in round one to 0.5% in round four. In the non-

intervention site (Koboko), no cattle infected with *T. brucei s.l.* were detected in cattle until Round 5 whereafter it ranged from 0.5% to 3.8% across three sampling rounds (Fig.5.7).

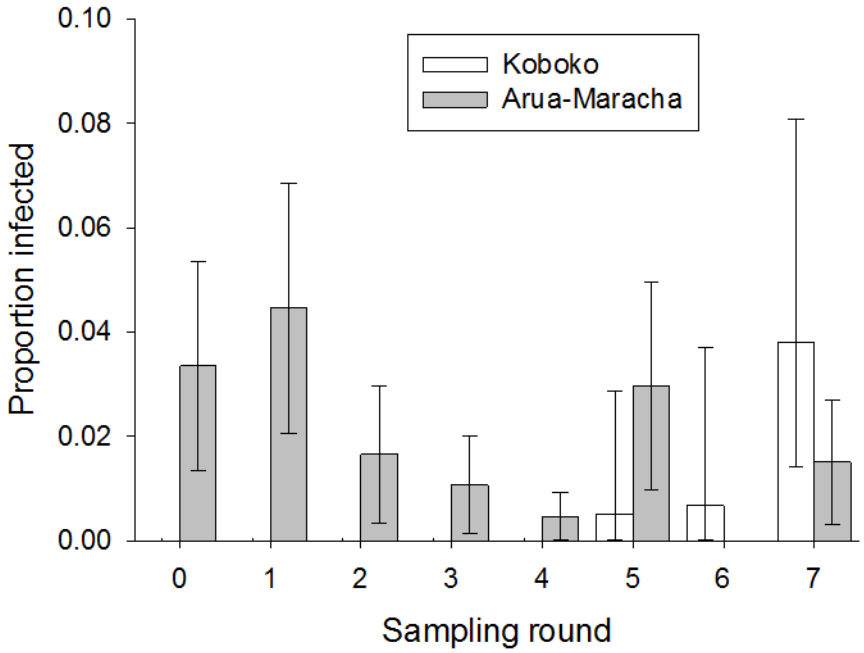


***Figure 5.8 The proportion of cattle infected with* T. brucei s.l.**
Positive cattle were identified using the Tbr-FIND primers across the control (Koboko) and intervention (Arua-Maracha) sites.

As with the microscopy results, newly recruited cattle could be contributing positive animals to the total being detected resulting in inaccurate reporting of infections acquired between sampling rounds. In the intervention site, when considering only animals that were present in consecutive sampling rounds, there was no decrease in the percentage of *T. brucei s.l.* infections from rounds one to three (1.3%-1.3%). From Round three to four, no cases of *T. brucei s.l.* were detected but from Round 4 to Round 5 the percentage of cattle positive for *T. brucei s.l.* was 3.6%. From round five to six no cattle were detected as positive however from round six to seven 1.9% of the animals tested positive. *T. brucei s.l.* positive cattle were not identified in the non-intervention site until round five (1%), the positive percentage remained the same in round six and increased to 2.2% in round seven. The general trend in prevalence across the sampling round does not decrease and there is no clear or consistent effect.

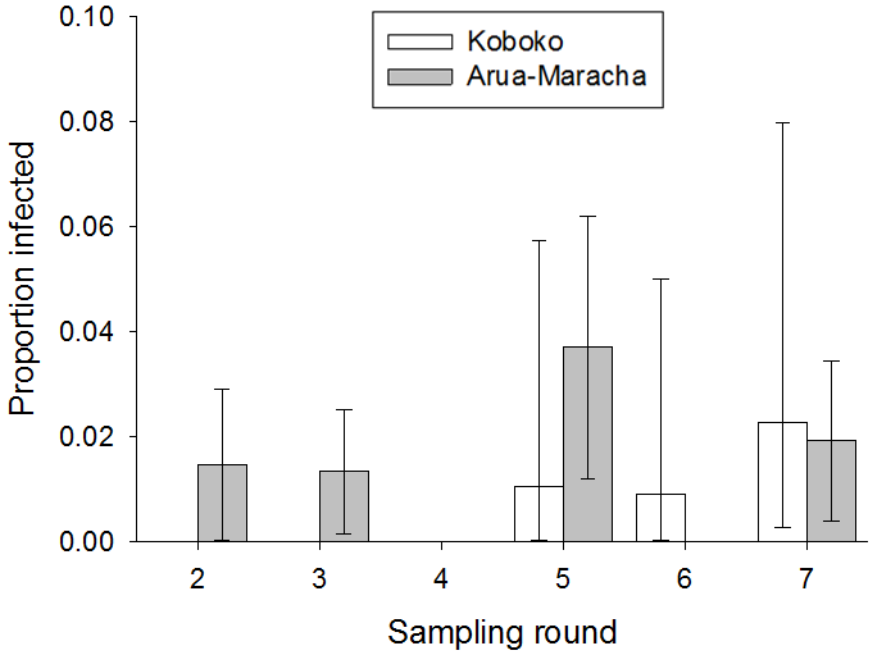


***Figure 5.9 Proportion of cattle infected with T. brucei s.l.*:**
Cattle sampled from consecutive sampling rounds

5.3.4 T. brucei s.l., T. congolense and T. vivax ITS positives

A total of 2,877 cattle samples were screened with the ITS PCR of which 293 (10%), were positive for either *T. brucei s.l., T. congolense* or *T. vivax* in single and mixed infections. *T. theileri* was found in 1,189 (41%) of all cattle screened with the ITS primers and in 197 (67%) of the 293 cattle that were positive for either *T. brucei s.l., T. congolense* or *T. vivax*. The total number of cattle samples identified as being trypanosome positive, be it salivarian or *T, theileri*, was 1440 (50%). When comparing single and mixed positives, only amongst the 293 salivarian positives, there were 275 (94%) single positives comprised of 125 (45%) *T. brucei s.l. ,* 53 (19%) *T. congolense* and 97 (35%) *T. vivax*. A total of 17 (6%) samples had a double salivarian infection of which 13 were mixed *T. brucei s.l.* and *T. congolense* infections, 3 were mixed *T. congolense* and *T. vivax* infections and 1 mixed *T. brucei s.l.* and *T. vivax* mixed infection (Table.5.3). Only one animal was positive for all three salivarian trypanosomes.

Table 5-3 ***The number of single and double salivarian positive samples identified.***

	Infections			
	Single	**Double**		
		T. brucei s.l.	*T. congolense*	*T. vivax*
T. brucei s.l.	125		13	1
T. congolense	53	13		3
T. vivax	97	1	3	

Comparison of the ITS results for *T. brucei s.l., T. congolense* and *T. vivax* for all cattle across the sampling period shows variation in the percentage of positive cattle in both intervention and non-intervention sites (Fig.5.9). Logistic regression analyses for each species separately or all species combined showed that the targets had no significant effect on infection rate.

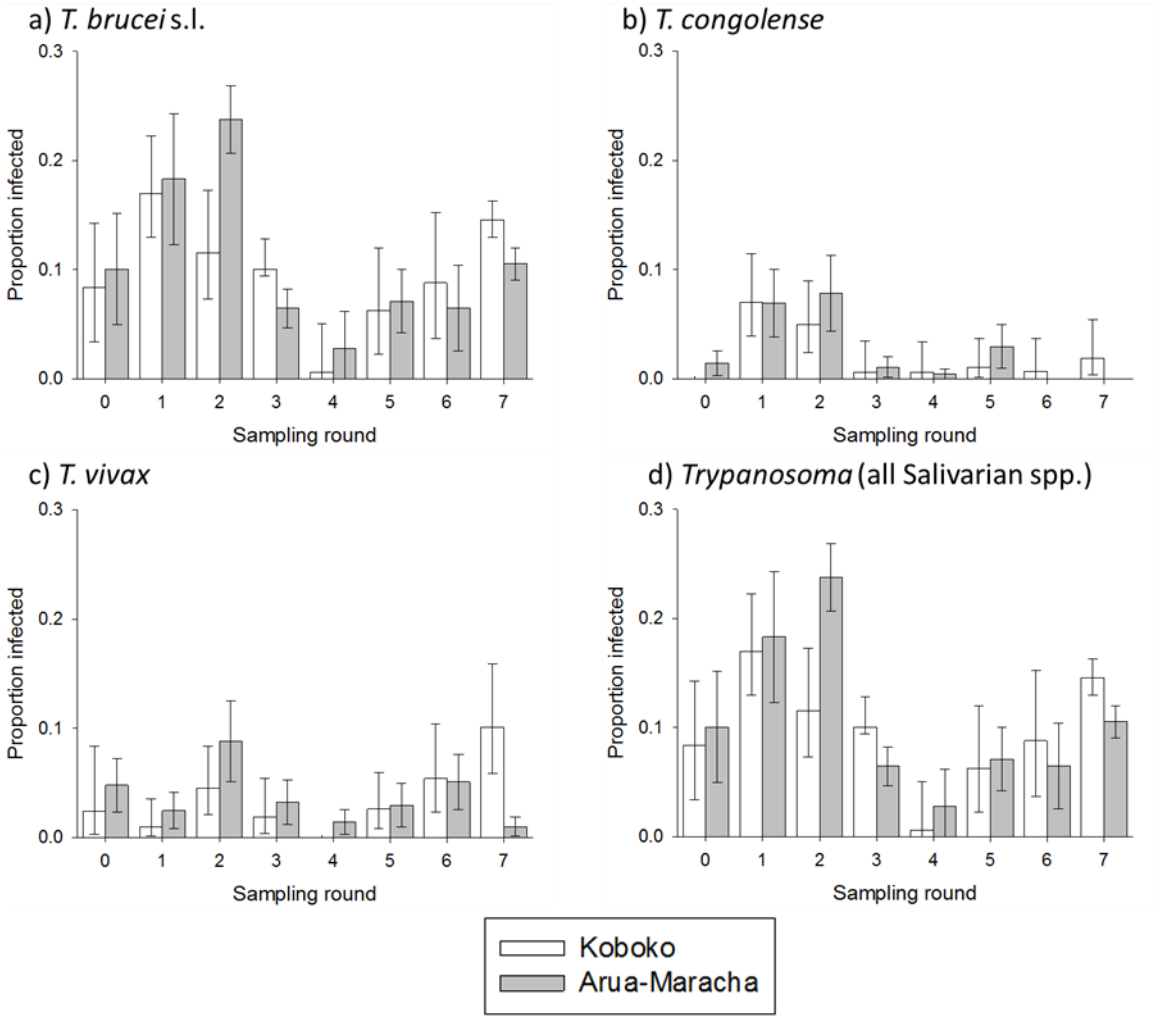


Figure 5.10 Proportion of cattle infected with **Trypanosoma** ***spp. assessed using the ITS PCR:***
(a) *T. brucei s.l.*, (b) *T. congolense*, (c) *T. vivax,* and (d) the combined infection rates for these three trypanosome species across all sampling rounds.

When comparing only animals that were present in consecutive sampling rounds (Fig.5.10) there was still no significant difference between the control and intervention sites for either *T. brucei s.l., T. congolense* and *T. vivax* or the combined salivarian species.

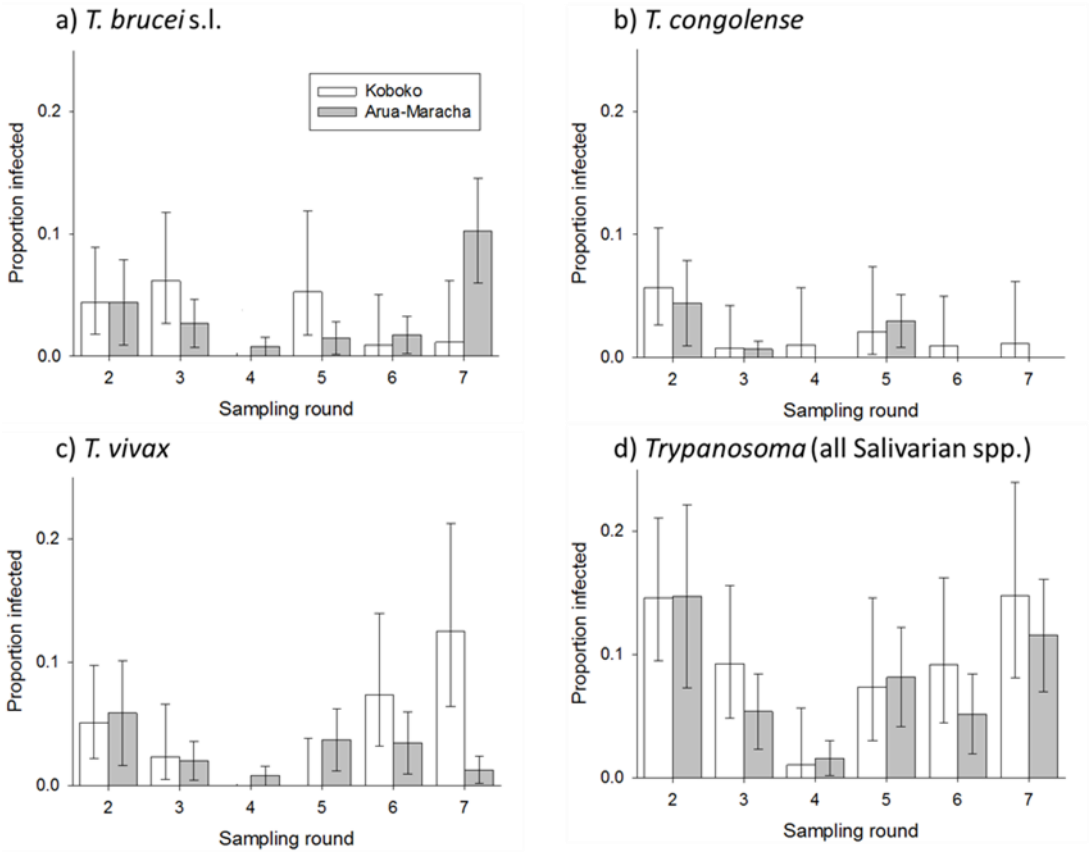


Figure 5.11 Infection rates estimated using the ITS PCR:
(a) *T. brucei s.l.*, (b) *T. congolense*, (c) *T. vivax*, and (d) the combined positive rates for all three trypanosome species from consecutive rounds.

5.3.5 ITS *T. theileri* positives

The number of samples positive for *T. theileri* from the intervention site was 581 (37%) out of 1,595 from the intervention site and 525 (40%) out of 1302 from the control site (Fig.5.11). There was no significant difference in the number of positives between the two sites across the sampling period (Fig.5.11a). However, there was a significant difference (GLMM, z=-2.19, P<0.05) for animals sampled from consecutive sampling rounds (Fig.5.11s.) with the proportion infected in the areas with and without targets being 0.41 (0.291-0.532) and 0.33 (0.219-0.470) respectively.

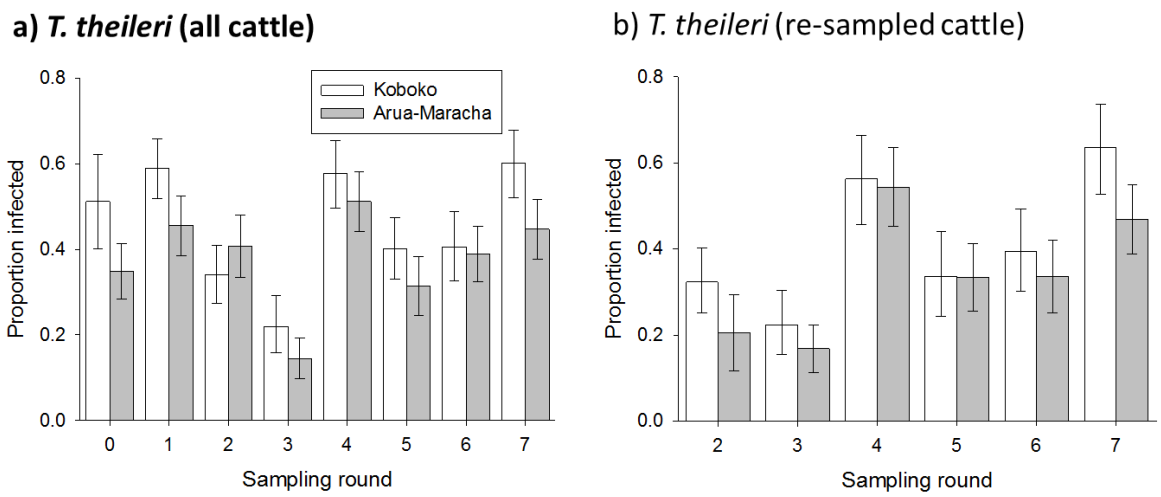


***Figure 5.12 The infections rates of cattle with T. theileri*:**
(a) all cattle and **(b)** only cattle that were re-sampled at consecutive rounds

5.3.6 Species specific primers for *T. vivax* and *T. congolense*

Of the 103 cattle identified as positive for *T. vivax* with the generic ITS primers, 27 were confirmed with the species specific *T. vivax* primer set described in Chapter 2. Of the 73 putative *T. congolense* positives identified with the generic ITS primers, none was confirmed with any of the three species specific primers used to identify the Kiliffi, Savannah and Forest/Riverine strains of congolense. This could either mean that the *T. congolense* samples identified by the ITS primers are not in fact *T. congolense* or that the lower sensitivity of the species specific primers is such that there is insufficient DNA present for them to work.

5.3.7 Sequencing results

To confirm the results from the species specific and ITS primers the single infections for trypanosome positive cattle were identified. For each species of interest, *T. brucei s.l., T. congolense* and *T. vivax* 10 samples were selected. This number was chosen as this was the maximum number of single *T. congolense* infections available. Of the 40 samples selected and processed with the 18S sequencing primers there was a total of 29 successful reactions (Table 5.4). The sequencing service was provided by Source Bioscience. As well as the salivarian species 11 suspected ITS *T. theileri* positives ranging in size from ~220-~380bp were also selected to confirm this parasite was as genetically diverse as suspected.

Table 5-4 ***Results of ITS and Tbr positives sequenced using 18S primers***

PCR		18S sequencing Results			
	Species ID by ITS and Tbr	***T. brucei s.l.***	***T. congolense***	***T. vivax***	***T. theileri***
Tbr	***T. brucei s.l.***	4	1		1
ITS	***T. congolense***	3	2		2
	T. vivax	1		1	3
	T. theileri	1			10

Although the species-specific primers had failed to identify the type of *T. congolense* present the sequencing primers amplified three *T. congolense* samples. When compared against the NCBI nucleotide collection database using BLAST search the *T. congolense* samples matched with *T. congolense* Forest with a 100% query cover and 99% identity.

5.3.8 *T. brucei* s.l. multiplex PCR and *T. b. gambiense* species specific PCR results

5.3.8.1 Tbr-FIND pig results

A total of 766 pigs were sampled from 13 sites (Fig.5.4), 400 pigs from seven sites in Arua district and 366 from six sites in Moyo district. When the pigs were sampled their colouration was recorded as an indicator of their breed. The local breed is characteristically smaller and black in colour with exotic breeds being white or mixed white and black and larger. Arua's local to exotic breed ratio was 3:1 and Moyo's was 1:7. The seven sites from Arua averaged 57 pigs and the six sites from Moyo averaged 61 pigs, Table 5.5.

Table 5-5 The Tbr-FIND results of pigs sampled from Arua and Moyo

District	Site	Easting	Northing	# pigs sampled	Tbr-FIND positive	Percent infected
Arua	**Wiliffi**	288042	364383	67	1	0.01
	Duku	292384	361141	45	1	0.02
	Tondolo	290083	354060	25	0	0
	Ngalabia	286723	351690	68	0	0
	Muttee	290934	360741	51	7	0.14
	Drimveni	289931	362178	100	3	0.03
	Inia	288042	364383	44	13	0.3
	sub-total			**400**	**25**	**0.06**
Moyo	**Perego**	359826	403404	37	0	0
	Opiro	359898	405066	109	0	0
	Moipi	357915	401930	67	0	0
	Kendi	357798	406514	113	0	0
	Goopi	363570	408774	32	0	0
	Kuleni	364337	407802	8	0	0
	sub-total			**366**	**0**	**0**
	Total			**766**	**25**	**0.03**

The number of pigs positive with the Tbr-FIND PCR assay was 25 (3.3%), all of these positives came from the Arua district; no pigs sampled from Moyo were found to be positive for *T. brucei s.l.*. Of the 25 positive pigs, 20 came from just two out of the seven sampling sites in Arua, these were Inia and Muttee. Sites Tondolo and Ngalabia produced zero positive pigs while Wiliffie, Duku and Drimveni produced the remaining five positive samples.

5.3.8.2 T. brucei species multiplex and TgsGP results

The *T. brucei s.l.* positive pigs and cattle were screened with the brucei multiplex primers to determine how many samples contained enough DNA for the amplification of a single copy gene. Of the cattle and pigs positive for *T. brucei s.l.,* 24 (63%) and 15 (60%) tested positively for the single PLC gene respectively. No samples were positive for the 669 bp sized band specific for the SRA gene which would indicate the presence of *T. b. rhodesiense*. The samples were then tested with the TgsGP primers to screen for *T. brucei gambiense*. Of the cattle and pig samples none was positive for *T. b. gambiense*.

5.4 Discussion

5.4.1 Effects of Tiny Targets

In this chapter multiple methods were used to identify trypanosome infections in cattle from two sites in NW Uganda that were in areas with or without Tiny Targets. The aim of the study was to assess the prevalence of Trypanosoma spp. in the cattle population and assess whether the deployment of Tiny Targets affected the incidence of infection in the local cattle population.

The intervention and control sites both lost cattle between sampling rounds which complicated the analysis as new cattle were recruited which confounded efforts to assess re-infection rates. This meant that during the analysis a distinction had to be made between the cattle sampled in consecutive rounds and those that had not. Both microscopy and molecular methods were used to identify the trypanosome positive animals. Microscopy identified trypanosome infection in 20% of the animals screened however the methods used were not suitable for accurately distinguishing between species and included animals infected with *T. theileri*. There was a significant difference in the number of microscopy positives between the two sites for individual sampling rounds and animals present in consecutive sampling rounds.

The results of the *T. brucei s.l.* specific primers identified 38 (1.3%) samples positive for *T. brucei s.l.* with an uneven distribution between the two sites. The intervention area contributed 30 Tbr positive cattle samples with only eight coming from the non-intervention sight. It was only in rounds five to seven that Tbr positive samples were found in the non-intervention site. These low levels are consistent with previous studies that found the prevalence of *T. brucei s.l.* ranging from 1.4%-10% (407, 408). Due to the uneven distribution of *T. brucei s.l.* and the low prevalence it is not possible to use the non-intervention site as a control to assess the impact of the Tiny Targets on reinfection rates of *T. brucei s.l.*.

Whereas microscopy identified trypanosomes in 20% of the samples, the generic ITS primers identified trypanosomes in 50% of all samples screened. The majority of these trypanosomes positives were *T. theileri* which was present in 41% of cattle samples.

Salivarian positives were found in 10% of cattle samples of which *T. brucei s.l.* was the most common. The generic ITS primers identified over three times as many putative *T. brucei s.l.* positives as the Tbr primers. The difference between the two PCR assays is unexpected as previous studies have shown that the Tbr PCR is more sensitive than the ITS PCR (253). A likely explanation for this is that the generic ITS primers amplified the ITS region of a trypanosome that shared a similar size to that of *T. brucei s.l..* Subsequently when run out and read on a gel it can be difficult to distinguish bands of similar size resulting in mis-identification. The species of trypanosome most likely to be producing these false positives is *T. theileri* as these trypanosomes, in this study, have produced ITS PCR products of ~380bp in size, similar to the 430bp size of *T. brucei s.l.*.

The ITS primers also identified *T. congolense* and *T. vivax* in which each contributed 24% and 35% towards the total number of salivarian positives. The regression analyses conducted on the sampling rounds found no significant difference between the intervention and non-intervention site with the exception of the analysis of animals from consecutive rounds positive for *T. vivax*. The result that *T. vivax* is the only salivarian trypanosome that differs significantly between the rounds is surprising as it has the shortest maturation time of the three salivarian species, 5-13 days, (295) and is known to be transmitted mechanically by tabanids (281). These life history traits make it less likely that *T. vivax* would be affected by tsetse control. Therefore, there is little evidence that the vector control implemented had any detectable impact on the reinfection rates of the study cattle. One explanation for this is that the majority of the *T. brucei s.l.* positives identified by the generic ITS PCR are potentially *T. theileri* wrongly identified as *T. brucei s.l.*. As *T. theileri* is not transmitted by tsetse but rather by tabanids, the Tiny Targets would have had little impact on the transmission of this parasite.

The lack of any clear and consistent impact of vector control on the incidence of Trypanosoma infections in cattle may be because of the mobility of tsetse and cattle. Infected tsetse from outside the intervention area may have moved into the area where targets were deployed and conversely cattle may have moved out of the area for grazing and water.

The species-specific primers for *T. congolense* and *T. vivax* confirmed 25% of the ITS *T. vivax* and none of the ITS *T. congolense* positives. Previous studies have also shown similar results with one study showing that the *T. congolense* species specific primers gave a prevalence of 0% compared to a prevalence 2.2% identified by the ITS primers (253). The results from the present study found an ITS *T. congolense* prevalence of 2.4% with a species specific primer result of 0%. The difference in prevalence of *T. theileri* between the intervention and control sites was found to be significant using binary logistic regression on animals present in consecutive sampling rounds. This difference is not due to the effects of the Tiny Targets but instead likely due to a pre-existing difference in the two regions.

5.4.2 Assessment the presence of *T. b. gambiense* in local cattle and pig populations

The second aim of this Chapter was to determine the prevalence of *T. b. gambiense* cattle sampled as well as in the local pig populations from two gHAT foci, Arua and Moyo. The identification of *T. b. gambiense* would show that transmission of sleeping sickness in the area is still on-going and the identification of the disease in either cattle or pigs would help resolve the role of animal reservoirs in the transmission of the disease.

The Tbr-FIND PCR analysis of the pig samples identified a 3% prevalence of *T. brucei s.l.,* this was double the percentage of cattle found to be positive with *T. brucei s.l.* (3%) using the same Tbr-FIND primers. All of the pig positives came from the Arua area, giving these samples a prevalence of 6%. A similar study, conducted in the same district in 2012, found pigs to have a higher prevalence (20%) of *T. brucei s.l.* (393). The results in this chapter have shown that *T. brucei s.l.* prevalences can be highly localised and this has been shown to be true in other studies (409). The method of blood collection and analysis between this study and that of Balyeidhusa was also different with the latter using venepuncture and 250µL of blood. It is therefore not possible to say that the difference in prevalence of *T. brucei s.l.* between this study and the study of 2012 is due to a decrease in overall prevalence of the disease. The lack of Tbr-FIND positives from Moyo district is harder to explain as the vector is the same as that found in Arua, *G. f. fuscipes* (328, 410) and it is an active foci of sleeping sickness transmission (406). One possible reason for the difference would be different animal husbandry techniques, although anecdotal, the pigs in Arua district were allowed to wander freely whereas the majority of pigs in Moyo district were kept in pig pens. The different methods of keeping pigs could reflect the fact that Moyo had recently suffered an

outbreak of swine fever and by penning their pigs they could limit the interaction the animals would have with each other. Another difference between the two sites was the proportion of local breeds with exotic breeds. Arua's local to exotic breed ratio was 3:1 and Moyo's was 1:7.

5.4.2.1 Reservoir animals

Previous studies from other parts of Africa had shown that a wide range of domestic animals were positive for *T. b. gambiense* (63-65, 395, 411). The importance of identifying the role these animals play in maintain transmission is critical if the goal of elimination by 2020 is to be achieved (394). To investigate this in the West Nile focus of Uganda, cattle and pig samples positive for *T. brucei s.l.* using the Tbr-FIND primers were screened with *T.brucei s.l.* sub-species specific primers and single-copy gene *Trypanozoon* primers. The single-copy gene primers were used to determine how many samples had sufficient DNA to detect down to a single-copy gene. This would allow the number of true *T. b. gambiense* and *T. b. rhodesiense* negatives to be identified, as opposed to those negative due to insufficient DNA for the PCR reaction to work (271). Of the cattle and pig Tbr-FIND positive samples, 63% and 60% had sufficient DNA to detect a single copy gene respectively. Of the samples with sufficient DNA, none was positive for *T. b. gambiense*. This supports the current evidence that pigs and cattle are not acting as reservoir hosts in NW Uganda (393). Different sampling methods could produce higher or lower prevalence estimates depending on how suitable they are for detecting trypanosomes. Typically, blood samples have been used for collecting samples for trypanosome identification, however recent evidence suggests that trypanosomes may be found in other tissues such as skin (412, 413) or fat (414). It may be that future surveillance programmes will need to screen different tissues to determine the most reliable sample to take.

5.4.3 Conclusion

It was not possible to detect any significant changes in the incidence of trypanosomiasis due to the deployment of Tiny Targets. This is most likely due not to the Tiny Targets being ineffective as it has been shown that the tsetse population decreased by >90% (236) but due to the subject animals leaving the protection of the intervention zone. The presence of *T. theileri* in a high proportion of animals and the use of generic ITS primers contributed to the inconclusive results. This is due to the fact that the re-infection rates of *T. theileri* were not affected by the intervention as it is not transmitted by tsetse and the presence of the parasite contributed to false positives when screening for salivarian trypanosomes transmitted by tsetse.

Chapter 6 : Discussion

6.1 Background

There are currently no vaccines or prophylactic drugs to control African sleeping sickness and this limits interventions to treat the human population. In their absence, vector control will be an important component of the global effort to achieve the targets of the London declaration, which seeks elimination by 2020 (415). Specifically, elimination of Gambian HAT has been defined as <1 new case of gHAT per 10,000 people in at least 90% of the disease foci, with a global incidence of <2000 new cases (416).

As the prevalence of disease decreases, cost-effective methods will be needed to identify areas of transmission to inform control efforts. Current strategies to control gHAT rely largely on active and passive screening and treatment of human population with relatively little vector control (417). By studying the tsetse population new control and monitoring methods could be introduced to supplement the current eradication efforts. Human screening and treatment clears the disease from local communities however without control of the vector the risk of re-infection remains the same, requiring constant surveillance and treatment. By targeting the vector population alongside treating human cases the disease is more effectively controlled. The vector population can also be used as an alternative to the human population, to identify areas of transmission (60).

It is within this context that this study has taken place in order to better understand a gHAT foci in its last stages prior to elimination. The NW of Uganda is a historical gHAT focus currently undergoing active control campaigns utilising both the traditional test and treat strategy as well as vector control (236). As such the prevalence of disease in this area has been steadily decreasing, with only four cases of gHAT being reported in 2015 (418).

6.1.2 Screening tsetse

Traditional microscopy based methods for trypanosome identification in tsetse limit the practicality of xenomonitoring. These methods are unable to identify the trypanosome to the species level accurately, this is especially true for immature infections where

trypanosomes are present in the midgut but it is impossible to identify the species. Equally if xenomonitoring methods are to be used they must be as simple as possible in order for them to be suitable for the field. This desired field-friendly quality rules out PCR, as it requires a fully equipped molecular laboratory (419). However, the recent developments in LAMP technology provide a possible alternative means of screening tsetse, as it has a better sensitivity and specificity than PCR as well as a simpler operating procedure.

6.1.3 Tsetse control

A tsetse control operation that results in a daily mortality rate of 4% is enough to drive the tsetse population to extinction (420) but it has been calculated that it only takes a drop of 72% in tsetse numbers to break gHAT transmission (236). Vector control has been successfully used to control Rhodesian HAT, where an effective vector control technology targets the *Morsitan* group (421). This same technology has performed poorly when used against the *Palpalis* group of tsetse. From 2007 to 2008 analyses of the responses of riverine species of tsetse to targets of various colour, shape and size, demonstrated that reducing the size of the traditional tsetse target increased its cost-effectiveness. This discovery resulted in the development of the Tiny Target, a novel control technology that successfully targets the *Palpalis* group of tsetse (231). The deployment of Tiny Targets in NW Uganda demonstrated the impact this technology has on the tsetse population, however the effect of this intervention on the transmission of *T. brucei s.l.,* and to a lesser extent on other salivarian trypanosomes, has not been investigated.

6.1.4 Animals as reservoirs

One potential problem that could hamper the elimination attempts of sleeping sickness is the role animals may play as disease reservoirs. Animals are an important reservoir host for *T. b. rhodesiense*, the pathogen which causes Rhodesian HAT, however the role of animals in the transmission of Gambian HAT is not fully understood (61, 63, 64, 422). The two domestic animals of interest in this study are cattle and pigs, both of which have been found infected with *T. b. gambiense* in other studies (391).

6.2 Chapter summaries and findings

6.2.1 Chapter 3: Prevalence of *T. brucei s.l.* and other *Trypanosoma spp.* in *G. f. fuscipes*

The vectorial competence of tsetse is dependent on a number of factors. These include: (i) host feeding preference, (ii) age structure of the populations and (iii) population size (423). Traditional dissection methods were used to estimate the age structure of the female population (424-426) as well as providing an initial assessment of the prevalence of trypanosome infection within the vector population. The species-specific ITS multiplex primers were employed to identify the prevalence *T. brucei s.l., T. congolense* and *T. vivax* in the vector population of Koboko. To assess patterns of blood feeding a subsample of tsetse midguts were screened with cytochrome B sequencing primers in order to identify vertebrate blood meals (199). Local weather data estimates were also used to try and identify their effects on the tsetse population.

6.2.1.1 Results of tsetse study

Tsetse were caught across a period of 16 months from April 2013 to July 2014. A correlation between rain fall estimates and tsetse catch numbers was observed when factoring in a lag time of two to three months between the rain fall data and tsetse catch numbers. The ovarian data revealed that the average age of tsetse across the sampling period fluctuated from a mean ovarian age category of 4.3 at the start of the wet season to 2.8 by the middle of the dry season.

6.2.1.2 Microscopy

Out of the 6664 flies caught from April 2013 to July 2014 along the Kochi river, 6,348 (95.3%) midguts, 6,399 (96%) salivary glands and 6,403 (96.1%) mouthparts were screened with traditional microscopy methods. In total, 6,335 tsetse had all three tissue types screened with microscopy and a further 73 had at least one tissue screened and 158 (2.5%) were positive.

6.2.1.3 PCR

A subsample of 2,184 tsetse were selected from the sampling period of September 2013 to February 2014. This date range was selected to include three months of wet season

(September-November) and three months of the dry season (December-February). A total of 194 (8.9%) of the flies were positive for either *T. brucei s.l., T. congolense* or *T. vivax* when the three tissue types (midgut, salivary glands and mouth parts) were pooled together. When individual tissues were re-screened it was possible to identify positive tissues in 126 (65%) of the 194 pooled positives. Of the 2,184 tsetse screened, 1.8% were *T. brucei s.l.* positive, 2.6% *T. congolense* positive and 2.2% *T. vivax* positive. The 126 positive tsetse resulted in 201 positive tissue samples of single or mixed infection.

6.2.1.4 Midgut

4.9% of 2, 183 midgut samples were infected with trypanosomes of which *T. congolense* was present in 2.2% samples, *T. vivax* in 1.6% and *T. brucei s.l.* was present in 1.2% of samples. The likely explanation of the relatively high number of *T. vivax* positive midgut samples is that this is due to trypanosomes from the mouthparts being flushed into the midgut with the bloodmeal as 67.6% of the *T. vivax* positive midgut samples had corresponding *T. vivax* positive mouthparts. Equally *T. vivax* trypanosomes could have been present in an ingested bloodmeal (268, 427).

6.2.1.5 Salivary glands

Salivary gland tissues were 1.5% positive comprising of 0.7% *T. congolense*, 0.6% *T. brucei s.l.* and 0.2% *T. vivax* positives. The most likely explanation for most of the salivary glands testing positive for *T. congolense* could be due to contamination, likely during the dissection of the tsetse fly. Evidence to support this is that of the 81% of *T. congolense* positive salivary glands occurred when *T. congolense* was also found in the midgut, mouthparts or both. This pattern of an increased likelihood of finding positive salivary glands when multiple tissues are positive does not hold with the *T. brucei s.l.* results. Of the 13 salivary glands found positive for *T. brucei s.l.* 62% came from tsetse where the salivary gland was the only positive tissue.

6.2.1.6 Mouthparts

Mouthparts had the second highest number of positives, after midguts, with 2.8% positive rate. The number of the mouthpart positives in this study per species were as follows: 1.6%

T. vivax, 0.9% *T. congolense* and 0.4% *T. brucei s.l.*. This distribution reflects what you may expect when using the Lloyd and Johnson method to determine the species.

6.2.1.7 Blood meals

Of the 768 midguts screened, with vertebrate cytochrome B primers (199), 17.1% produced a vertebrate host sequence, 8.2% from the wet season and 8.9% from the dry season. Cattle contributed 39% samples to the overall total. Human blood meals were second with 48 (37%) positives. The third and fourth most common blood meals identified came from the Nile monitor lizard and forest cobra with a total of 19 (15%) and 5 (4%) respectively. These four host types made up 94% of the blood meals the remaining 6% of blood meals identified were pigs, goats, sheep and a bird. There was no significant variation in proportions between the two seasons with regards to feeding rates on different hosts. This lack of significant variation in blood feeding has been found in previous studies (428).

6.2.2 Chapter 4: LAMP as a field based xenomonitoring tool

There are currently no highly sensitive and specific techniques for identifying trypanosomes in tsetse that are suitable for xenomonitoring in the field. The advent of isothermal molecular assays have the potential to provide an alternative to dissection and PCR through technologies such as LAMP (150, 154, 155, 429, 430). A diagnostic kit designed to detect *Trypanozoon* infections in human patients was tested for its suitability in detecting *T. brucei s.l.* from tsetse tissues.

6.2.2.1 Simplification of DNA extraction

A simple boil and spin DNA extraction method using 5% chelex was developed and tested with *T. brucei s.l.* positive and negative tsetse samples pooled together. The ratio of positive to negative tsetse midguts tested was 1:5, 1:10 and 1:20. Apart from two instances of kit failures the LAMP kits were able to identify the positive midgut, even in a 1:20 ratio.

6.2.2.2 LAMP kit limit of detection

To test the limit of detection of the LAMP kits a 1 in 10 dilution gradient of lab cultured *T. b. brucei* (J10) was added to tsetse midguts. The dilution ranged from $1x10^5$ to 0.01

trypanosomes per mL and the experiment comprised of six replicates for each of the eight dilutions. The results showed that the LAMP kits had a higher degree of sensitivity, with a third of the replicates being able to detect 0.1 trypanosomes per mL whereas the Tbr-FIND primers were unable to detect anything with a concentration of <10 trypanosomes/mL.

6.2.2.3 Persistence of trypanosome DNA

As the focus of the proposed Xenomonitoring method relied on the detection of DNA, it was important to ascertain how long after ingestion by the tsetse the target DNA could be detected. The experimental set up involved heat killing 200µL of trypanosome stabilate at 54ºC for 15 minutes prior to adding them to 4.8mL of defibrinated horse blood. The final concentration of trypanosomes in the horse blood was $4x10^4$ per mL (200uL at $1x10^6$ added to 4.8mL). The average volume of blood consumed by tsetse from the colony at the LSTM is 30µL, meaning that the average number of dead trypanosomes consumed by a single tsetse would be 240 (5,000µL divided by 30µL= 166.7, trypanosomes concentration of $4x10^4$ divided by 166.7 = 240.). After 48hrs it was still possible to detect *T. brucei s.l.* in all experimental flies, by 72hrs seven out of nine were positive. The number of positive tsetse decreased and after 144hrs post blood meal only one out of nine midguts tested positive for *T. brucei s.l.*. The DNA persisted in the tsetse midgut for a considerable amount of time despite the tsetse being fed a fresh blood meal every 48hrs.

6.2.2.4 Specificity of the LAMP kit

To test the specificity of the LAMP kits, tsetse from the colony at LSTM were prepared with single and mixed infections of *T. b. brucei* (J10) and *T. congolense* (1/148). The infection status was checked with microscopy, Tbr-FIND primers and generic ITS primers. The LAMP kits showed no cross reaction with the single *T. congolense* infections and were still able to identify the *T. brucei s.l.* positive tsetse in the mixed infections. The cross reactivity of the kits was tested further with 449 wild caught tsetse that had been screened with the generic ITS primers which had identified a broader range of trypanosome species than just *T. congolense* and *T. brucei s.l.*. Once more the LAMP kits did not cross react with the non-target species and identified both the ITS *T. brucei s.l.* positives plus four more.

6.2.3 Chapter 5: Impact of tiny target intervention on trypanosome transmission

The implementation of a new tsetse control technology across 500km^2 of NW Uganda has successfully reduced the tsetse numbers by >90% (236). Local cattle from both the intervention site and an ecologically similar control site were sampled from August 2011 to November 2013. The sampled cattle were treated with the trypanocide, Veriben, to clear them of infection in order to assess how many became re-infected between sampling rounds. The blood samples were initially screened in the field with microscopy using wet preparations and then subsequently screened using species specific Tbr primers, generic ITS primers and 18S sequencing primers at the LSTM.

6.2.3.1 Microscopy results

The microscopy work was carried out in the field using wet preps taken from the buffy coat layer of blood sample that had undergone centrifugation for PCV readings. A total of 3,173 samples were screened using this method of which 21% were positive. The percentage of animals positive for the intervention and non-intervention sites was 17% and 26% respectively. There was no significant difference in the prevalence of *Trypanosoma* spp. in cattle from areas with or without Tiny Targets.

6.2.3.2 T. brucei s.l. positive results

The results for *T. brucei s.l.* differed between the Tbr-FIND primers and generic ITS primers, with 38 detected by Tbr-FIND and 141 with ITS. The distribution of the Tbr-FIND positive cattle was not even between the intervention and control sites, with 30 positives being detected in the intervention site and eight in the control site. The distribution of ITS *T. brucei s.l.* was 79 in the intervention site and 62 in the control site.

6.2.3.3 T. brucei s.l., T. congolense and T. vivax ITS positives

The prevalence of the three salivarian species of interest for all cattle screened was 10% of which 94% were single infections. *T. brucei s.l.* positives were the most prevalent making up 45% of single infections followed by *T. vivax* at 35% and *T. congolense* at 19%. Only 6% of the positive samples were double infections, the most common being mixed *T. brucei s.l.* and *T. congolense* (76%) followed by *T. congolense* and *T. vivax* (13%) and lastly *T. brucei s.l.* and *T. vivax* (6%). Only one animal was found to be infected with all three salivarian

trypanosomes. When comparing the result from the intervention and non-intervention sites there was no significant difference in the prevalence of the different salivarian species in all cattle sampled. When analysing only those animals present in consecutive rounds only *T. vivax* showed a significant difference between the two sites.

6.2.3.4 T. vivax and T. congolense species specific primers

Samples positive for both *T. vivax* and *T. congolense* were re-screened with species specific primers in order to confirm the results however only 27 out of a total of 103 ITS *T. vivax* samples were confirmed with species specific primers and no *T. congolense* positive ITS samples were positive with any of the three species specific *T. congolense* primer sets.

6.2.4. T. theileri

The ITS PCR results revealed a high proportion of *T. theileri* infections in the cattle, out of 2877 cattle screened 1,106 (38.4%) were positive. The numbers of *T. theileri* fluctuated but remained relatively stable and was significantly different ($P<0.05$) between areas with and without Tiny Targets. T*. theileri* is not transmitted by tsetse and so the difference was unexpected and unexplained.

6.2.5 Chapter 6: Looking for *T. b. gambiense* in *Glossina* and domestic animals

The purpose of this chapter was to try and identify the presence of *T. b. gambiense* from the vector population as well as local domestic animals sampled from HAT foci in NW Uganda. The *T. brucei s.l.* positive tsetse samples from chapter 3 and cattle samples from chapter 5 were included. Pigs were chosen as a secondary domestic animal to screen for *T. b. gambiense* as they had been recorded with a high incidence of *T. brucei s.l.* from a previous study (393). To this end 766 pigs were sampled across two HAT foci from Arua and Moyo districts from areas that had previously reported sleeping sickness cases. These pig samples were processed in a similar manner to the cattle samples but were screened only using the *T. brucei s.l.* specific Tbr-FIND primers.

6.2.6 prevalence of *T. b. gambiense* and the role of animal reservoirs

The role of domestic animal reservoirs in the transmission of *T. b. gambiense* has not been definitively answered. To further investigate this question the Tbr-FIND positive cattle and

pigs were re-screened using primers specific for both *T. b. gambiense* and *T. b. rhodesiense* as well as a universal single copy *Trypanozoon* target. To collect the pig samples the sleeping sickness foci of Arua and Moyo were targeted for blood samples taken from ear vein-punctures. A total of 766 pigs were sampled from both sites, 400 from Arua and 366 from Moyo, The *T. brucei s.l.* positive tsetse samples were also screened for *T. b. gambisense.*

6.2.6.1 T. brucei s.l. positives in tsetse, cattle and pigs

Within the NW sleeping sickness foci of Arua and Koboko, 1.8% of tsetse tested were positive for *T. brucei s.l.* using the multiplex ITS primers and of the cattle samples screened with PCR 5% were positive for *T. brucei s.l.* by ITS and 1.5% by Tbr-FIND PCR. The results from the Tbr-FIND assays were considered to be more reliable than those from the generic ITS PCR and only these were screened with the HAT specific primers. The pig survey resulted in 3% positive rate in pigs for *T. brucei s.l.*, all 25 positive pigs came from Arua whereas no pigs from Moyo were found to be infected.

6.2.6.2 T. brucei multiplex and sub-species specific PCR

Following on from the *Trypanozoon* multiplex PCR 24 (63%) of cattle, 15 (60%) of pigs and 25 (56%) of tsetse samples had enough genetic material for the species specific primers. These samples were then re-screened with the *T. b. gambiense* specific TgsGP primers and *T. b. rhodesiense* SRA primers. Both the cattle and pig samples were negative for both the Gambiense and Rhodesiense sub-species of *T. brucei.* The tsetse samples were negative for *T. b. rhodesiense* but 16 samples produced a faint product which appeared to be close to the 308bp size expected of the TgsGP primers. Three of these samples were sent for sequencing alongside the positive control. The results from the positive control resulted in a 100% query cover and identity match with *T. b. gambiense* however the three samples returned a different sequence. The sequence returned from all three samples was the same and failed to match any on the NCBI database.

6.3 Research questions revisited

This thesis has examined four research questions that relate to the transmission and control of *T. brucei s.l.* in the historical HAT foci of the West Nile region of Uganda within the context of the elimination goals of the London declaration.

6.3.1 What is the prevalence of *T. brucei gambiense* in the tsetse population?

The NW of Uganda is known to be a historic focus of gHAT (56), however the factors that contribute towards maintaining these foci are not well understood (431). Despite being understood as the sole vector of *T. b. gambiense*, the pathogen causing gHAT (161), the prevalence of the disease amongst wild tsetse population is often extremely low (391, 432, 433) and attempts to infect tsetse with *T. b. gambiense* under laboratory conditions have often proven unsuccessful (434). Modelling studies have suggested the prevalence of *T. b. gambiense* in the wild may be as low as 1/4000 flies (435) and in light of this evidence the role of tsetse in gHAT has recently been called into question (436). The need to establish the prevalence of *T. b. gambiense* is therefore critical in understanding the role of the vector in the gHAT foci of NW Uganda and what interventions are required in order to achieve elimination by 2020.

The prevalence of *T. brucei s.l.* in the tsetse population of NW Uganda was investigated in Chapter 3 and differed between the microscopy and PCR results. Microscopy results could only identify a mature *T, brucei s.l.* positive whereas the mITS PCR assay was able to identify both immature and mature *T. brucei s.l.* infections. Using microscopy 0.11% of the tsetse were identified as having a mature infections by observing trypanosomes in the salivary glands (135). This low prevalence is consistent with previous studies (135, 203). The mITS PCR reactions identified *T. brucei s.l.* DNA in 2% of the tsetse screened, of which 0.6% were salivary glands, indicating a mature infection. In order to answer the question of *T. b. gambiense* prevalence in the tsetse population of the historic gHAT foci of NW Uganda, the samples identified as *T. brucei s.l.* positive by mITS were then re-screened in Chapter 6 with sub-species specific primers. None of the samples tested positive for either of the trypanosomes responsible for HAT. A product was produced by the TgsGP primers but this was smaller than the expected *T. b. gambiense* product and when its sequence was

analysed it did not match with that of anything recorded on the NCBI online database. The lack of any tsetse infected with *T. b. gambiense* out of a total of 2, 184 tsetse is in keeping with previous results from wild tsetse surveys. This apparent lack of *T. b. gambiense* in NW Uganda could well be down to the number of tsetse screened, as the number was <4,000 calculated by the model and the incidence of disease is decreasing in the area with only four cases reported in 2015 (418). The low sensitivity of the methods used greatly reduce the chance of identifying the few tsetse positive for *T. b. gambiense.*

The alternative explanation for the lack of *T. b. gambiense* positive tsetse samples is that, in the areas sampled, there is currently no active transmission of the disease. Due to the chronic nature of the disease the four individuals positive in 2015 for gHAT could well have been infected years before the tsetse sampling conducted in this study.
There are only two possible explanations for the non-target product amplified by the TgsGP primers. Either it is a true result and an unknown strain or sub-species of trypanosome was identified as a member of the *Trypanozoon* sub-species group by the mITS primers and shared enough similarities with *T. b. gambiense* to produce a PCR product when screened with the gHAT specific primers, or it is a false result due to non-specific binding to DNA resulting in the amplification of a random product. Attempts to sequence the sample were hampered by the presence of *B. saltans* present in the sample. Due to time constraints it was not possible to investigate the non-target product further.

6.3.1.1 Constraints of the study

The number of tsetse screened with the mITS primers may not have been large enough as the modelling studies suggest the prevalence of *T. b. gambiense* in tsetse could be 1 in 4,000 (435). There is also the issue of sensitivity with the sub-species specific primers being unable to identify low prevalences of target species. An option of increasing the yield of trypanosome DNA would be to have cultured any microscopy positive tsetse to increase the final DNA yield collected from the sample (437). The ability to sequence the samples was compromised due to the contamination of samples by *Neobodo designis* which was introduced when contaminated water was used in the preparation of PBS for dissection. The generic nature of the 18S sequencing primers would often result in the amplification of *N. designis* over any salivarian trypanosome present DNA. The ITS multiplex primers were

designed to help focus the study to the most common species of salivarian trypanosomes that were likely to be found and reduce the confusion that the generic ITS primers caused, however due to this increased specificity other salivarian species of trypanosome such as *T. simiae* and *T. grayi* would not be observed. The inclusion of more trypanosome species could help uncover co-infection patterns which could be used to further understand the transmission of the disease. Issues of comparative sensitivity between the two tests were alleviated with the use of the same outer primer in both nests thereby requiring the inner primers be designed from the same internal sequences which will have comparative copy numbers across both primer sets for each species.

Despite the improved specificity of the multiplex ITS reaction both PCR methods still rely on the use of gel imaging to process the results. Gel imaging itself can present problems from warped gel combs, uneven gel runs, sub-optimal UV light boxes and issues with determining accurate band sizes. A nested PCR reaction improves the sensitivity of the assay but also increases the risk of contamination as the reaction tubes from the outer nest are opened post amplification of DNA in order to seed the inner nest.

6.3.1.2 Future Research

What is the identity of the DNA that resulted in the non-target TgsGP ?

An unknown DNA template has successfully cross-reacted with a highly specific primer set designed to identify *T. b. gambiense* the parasite responsible for gHAT. Identifying the source of the DNA template would determine if the results observed are true results, in which case an unknown trypanosome cross-reacted with the TgsGP primers, or false results.

By further analysing the human blood meal samples is there a difference between the sexes with regard to feeding frequency?

The roles of men and women typically differ in the rural communities of NW Uganda. These differences in behaviour may result in different exposure risks to tsetse bites and subsequently gHAT. Using sex-specific primers (438, 439) it would be possible to identify what proportion of the human blood meals identified in Chapter 3 are male or female and whether or not there is a risk factor associated with gender.

6.3.2 Can commercial LAMP kits be used for xenomonitoring?

One of the study limitations conducted in Chapter 3 is that the number of tsetse screened for *T. b. gambiense* may be too small. If a larger number of tsetse need to be sampled then a different approach to sampling may need to be employed. Microscopy is more pragmatic for use in the field however lack sensitivity, LAMP provides an alternative to either microscopy or PCR. Such an approach may also be more suited to the detection of gHAT transmission sites where control measures have successfully driven down the prevalence of disease (53).

The assessment of the LAMP kits showed that they would work on DNA samples that had undergone a simplified extraction process and that the LAMP tests were extremely sensitive, being able to detect the equivalent of 0.1 trypanosome per mL. The ability of the LAMP kits to detect *Trypanozoon* DNA in the midgut of the tsetse showed that after 48hrs the trypanosome DNA was still detectable in 100% of the samples and even after six days it was still possible to detect the *Trypanozoon* DNA in 1/9 samples.

Whereas the microscopy is limited to only being able to detect identify *T. brucei s.l.* if there is a mature salivary gland present molecular methods are capable of detecting DNA, even from transient infections. Therefore, xenomonitoring of tsetse using LAMP is not going to just identify those tsetse with an active mature infection, but rather any tsetse that has detectable DNA from either a bloodmeal or transient infection. This will, in theory, increase the chances of detecting *T. b. gambiense* DNA in the tsetse population.

The LAMP kits have also been shown to work well with pooling samples together. In the current study the maximum number of samples pooled was 20. If the model data is correct, and ignoring the fact that xenomonitoring does not need to identify an active infection, the likelihood of detecting a *T. b. gambiense* positive tsetse is 1/4000 (435). By pooling the samples in groups of 20 this would reduce the number of reaction down to 200. It may be possible to reduce this down further as this study did not identify the limits of pooling tsetse samples. As the prevalence of gHAT continues to decrease the surveying approach currently employed will no longer be suitable as the number of cases will be so low no sampling of the human population will have enough statistical power to inform the decision making process (53). The application of xenomonitoring may provide a valid alternative in checking areas for the presence of gHAT towards the late stages of the elimination campaign.

6.3.2.1 Constraints of study

Although proven to work under laboratory conditions this work has yet to be carried out in the field. There is also the disadvantage that the LAMP kits are only able to identify *Trypanozoon* positive tsetse and a further sub-species specific test would need to be run to confirm the presence of *T. b. gambiense*. Currently the only assay capable of doing this relies on the single copy TgsGP primer. Therefore, the limitations for detecting other *T. b. gambiense* positives still apply. There is also the issue of assessing the limits of pooling samples, currently the largest pool used is 20 however it may be possible to use larger pools, so as to reduce the number of test required to identify any positive *T. b. gambiense* tsetse.

6.3.2.2 Future research questions

What are the pooling limits of the LAMP kits?

With the potential prevalence of *T. b. gambiense* in the tsetse population potentially being as low as 1 in 4,000 a larger pool of samples would help in screening a large enough sample size faster and with fewer resources. It would therefore be advantageous to identify what is the maximum pooling size to use with the LAMP assay.

How practical are the methods developed in Chapter 4 for use in the field?

Chapter 4 has shown that the LAMP assay works well in detecting *T. brucei s.l.* infected tsetse, however this was conducted in a well-equipped laboratory using colony reared tsetse. In order to properly assess the suitability of this method it would need to be conducted in the field.

What are the health economics of xenomonitoring gHAT foci with LAMP kits compared to standard screening campaigns of the human population?

The currently there is no analysis of the cost benefit of using the LAMP xenomonitoring strategy studied in Chapter 4. In order for this method to be a viable alternative to screening the human population it would need to be able to identify *T. b. gambiense* in the environment at a lower cost than the current methods used.

6.3.3 Do 'Tiny Targets' affect the prevalence trypanosomes in the local cattle?

The deployment of Tiny Targets in NW Uganda has been shown to reduce the tsetse population by >90% and it has been calculated that a drop of 72% in the tsetse population is sufficient to break transmission (236). Therefore, based on the drop in tsetse catch numbers from the NW Uganda following the tiny target intervention one would expect to see a decrease in the prevalence of cattle infected with salivarian trypanosomes. However, this expected drop in infected cattle from the intervention site of Arua was not observed in either the microscopy results or in the PCR results, with the exception of cases for *T. vivax.* The design of the study required the intervention and non-intervention sites to be revisited across the study period (August 2011-December 2013) and for the cattle sampled and treated in the previous round to be re-sampled and treated. This ensures that at each sampling round animals are cleared of infection allowing for reinfection rates to be observed. The number of cattle lost between rounds was ~30% with both sites losing similar numbers of cattle. Therefore, the most accurate results for reinfection rates are from the animals present in two consecutive sampling rounds rather than comparisons between all animals in each sampling round. The analysis of microscopy results from animals present in consecutive rounds did not show a significant difference, between the intervention and non-intervention sites. Morever, analyses based on the generic ITS PCR results found no significant difference in the total salivarian prevalence between the two sites during the sampling period. The samples were also processed with the highly sensitive and specific Tbr-FIND primers to identify the prevalence of *T. brucei s.l.* as an alternative to the generic ITS primers. These results showed a significant difference between the two study sites with the majority of the *T. brucei s.l.* positives coming from the intervention site and only appearing in the non-intervention site in round four. Despite having a lower limit of detection the Tbr-FIND primers detected more than three times fewer *T. brucei s.l.* positives. The reason for this is discussed more extensively in Chapter 5 but essential it is most likely due to mis-identification due to similar sized *T. theileri* PCR products.

T. theileri, as identified by the generic ITS primers and the 18S sequencing primers, was present in 41% of the 2,877 cattle screened with PCR and most likely contributed towards the majority of the microscopy positives as the parasite is very large and is easier to see than the much smaller salivarian species. The analysis of the ITS PCR results showed a

significant difference in the number of *T. theileri* cases between the intervention and non-intervention sites with the latter having a higher incidence of positives. *T. theileri* parasites were also present as a co-infection in 60% of the salivarian positive cattle.

6.3.3.1 Constraints of study

The Tiny Targets have been proven to drastically reduce the number of tsetse and maintain this reduction so long as the targets are regularly maintained. Barring the results for *T. vivax* there was no significant difference between the two study sites with regards to re-infection numbers. This is likely to be caused by the cattle leaving the protection of the intervention sites as they are taken out to graze and water. We had no methodology setup whereby we could track the movements of the cattle and either prove or disprove this theory by showing them leaving the protection of the intervention sites.

The alternative would be to have a cohort of animals that belonged to the study and were under its control in order to ensure the animals never left the intervention sites. The cost of cattle and their general need to graze results in these animals being ill suited for such a role. Pigs would be a better candidate for a more stationary cohort of animals as they are cheaper than cattle and they possessed a higher *T. brucei s.l.* positive infection rate compared to cattle. The major disadvantage of using pigs is that they are not found in the control site in large enough numbers due to the majority of people in Koboko being Muslim. Another potential downside to using pigs could be their relatively high turn-over rate compared to cattle.

A second limitation relates to the approaches used to identify the trypanosome species in the cattle. With regards to the microscopy screening no steps were taken to measure the size of the trypanosome which would have allowed for the distinction between *T. theileri* and salivarian infections in cattle as the average size of *T. theileri* is 47.2µ (133) whereas the salivarian species range in size from 9-33µ (440). The generic ITS primers were also not well suited for the purpose of screening the cattle as they amplified the *T. theileri* parasites resulting in confusion of species identification based on PCR product size.

A better solution would have been to design and screen the samples using qPCR ideally TaqMan as it would have been possible to design generic primers but use a multiplex of different probes to be able to detect multiple, sequence specific, targets. The number of probes would be limited by the number of light channels available in the machine. Currently at LSTM there are machines available with five light channels, this would be sufficient to target *T. brucei s.l., T. congolense*, *T. vivax* and two extra, potentially *T. simiae* and *T. grayi*. Or alternatively one of the extra light channels could be used as an internal positive control to ensure there are no inhibitors in the reaction, a suitable candidate for this would be the seal herpesvirus (441, 442). The use of TaqMan would also allow for the quantitation of parasite DNA loads which would be an additional element of enquiry, naturally it may be hard to make a direct correlation between interventions and Ct values due to the fluctuating parasitaemia.

The method of sample collection could also have been a limiting factor as venous blood was collected from the ear in order to screen for trypanosome infection but very recent studies have shown that the skin may be an important anatomical reservoir for salivarian trypanosomes (413).

6.3.3.2 Future research question

How suitable are different sampling methods in detecting the prevalence of salivarian trypanosome infections?

Current sampling methods for diagnosing the hemolymphatic stage of trypanosome infections rely blood from either a pin-prick or veinous extraction. These can then be screened in a number of ways such as microscopy, PCR and serology, to try and identify the presence of trypanosomes. The confirmation that skin may be an important reservoir tissue for trypanosomes raises the question of how suitable are skin samples, such as skin-snips, in the screening of mammalian hosts for trypanosome infections.

Could ticks be a suitable alternative to current blood sampling methods for the detection of Salivarian trypanosomes in animals?

Ticks are common parasite on the cattle in Uganda and feed in a similar manner to tsetse flies, by pool feeding (443). This method of feeding involves the laceration of tissues around the bite wound including the capillaries in order to generate a pool of blood from which to

feed. This disruption of the skin followed by the introduction of anticoagulants and subsequent blood feeding may result in an increased chances of trypanosome uptake by a tick as compared to the use of a lancet and for pin-prick extraction of blood from the ears of cattle. This is a similar concept to the use of tsetse in xenomonitoring, in that the tick does not need to go on to develop a mature infection rather the presence of a trypanosome within the digestive tract would be sufficient to allow detection with a molecular assay such as PCR.

What are the normal movements of cattle, where do they graze and water?
A key piece of information missing from the study described in Chapter 5 is the movements of the cattle in the tsetse infested areas. Knowing the movements of cattle would help better understand the results generated in the Chapter.

Based on the blood meal results from Chapter 3, cattle are the primary hosts in the area, could they be used as a tsetse control by being treated with insecticide?
The tsetse blood meal results from Chapter 3 showed that the majority of blood meals originated from cattle. This implies a high interaction between the tsetse and local cattle populations raising the possibility that insecticide treated cattle could be used as a complementary vector control alongside the Tiny Targets.

6.3.4 Is *T. b. gambiense* present in the cattle and pigs from the gHAT foci in NW Uganda?

Modelling studies show that the success or failure of meeting the sleeping sickness elimination goal of 2020 will depend on a number of parameters. An animal reservoir host has been shown to delay and even prevent the elimination of gHAT using the current strategies (61, 394, 435). The presence of an animal reservoir also changes the importance of the other parameters. Without an animal reservoir the most important factor in transmission of the disease is the number of people commuting to an area with a high tsetse biting rate, however with an animal reservoir the importance of this changes and the ratio of tsetse to humans becomes the most important parameter (435). Therefore, to be able to identify an animal reservoir would be important in informing and developing future control strategies. The identification of *T. b. gambiense* in wild and domestic animals (63, 64, 391) supports the theory of a potential reservoir host as does the presence of the

parasite in the vector population in an area with no human cases (60). This chapter aims to try and answer this question for the foci in NW Uganda. A previous study had been conducted in this area (393) however it is not clear where the sampling took place and as the disease distribution is highly focal it is not clear if the sampling had occurred in the correct place. Our study aimed to sample animals from areas that had recently reported gHAT cases (406). Based on the results of the blood meal analysis from Chapter 3 in which cattle featured as the most common host the Tbr-FIND positive cattle from Chapter 5 were screened for *T. b. gambiense*. Alongside the cattle, pigs were also chosen as a potential reservoir host based on evidence in the literature of high *T. b. gambiense* infection rates (64, 65, 395) and also on the blood meal analysis from Chapter 3, as pigs were the third most common mammalian host despite comprising only ~0.1% of domestic animals in the area.

A total of 766 pigs were sampled for this study from two districts with a high incidence of gHAT, Arua and Moyo. The samples were processed in the same manner as the cattle samples resulting in 3% of the pigs identified as *T. brucei s.l.* positive using the Tbr-FIND primers. The cattle and pig *T. brucei s.l.* positives were then screened with the *T. brucei*-multiplex primers (271) to determine if there was sufficient *Trypanozoon* DNA present for single copy gene detection and to rule out the presence of *T. b. rhodesiense*. Following this assay the samples were processed using the *T. b. gambiense* sub-specific primers (272). This final assay found no *T. b. gambiense* positives in either the pig or cattle samples. This corresponds with the finds of the previous study and supports the notion that in the HAT foci of NW Uganda neither cattle nor pigs are acting as a reservoir host for gHAT. The implications for this finding are that the current control methods, case-detection and treatment of the human population combined with the new Tiny Target vector control technology are sufficient to eliminate gHAT from this foci.

6.3.4.1 Constraints of study

This study suffers from similar limitations to those mentioned in the first question regarding the sensitivity of the TgsGP primers. Of all the *T. brucei s.l.* positives in both cattle and pigs ~40% lacked enough DNA material for the detection of single copy genes. Therefore, for these samples it is not possible to determine which species of *T. brucei s.l.* is present.

Another weakness of the study is that the numbers used for both pigs and cattle may not have been large enough for the purpose of the study resulting in it being underpowered.

6.3.4.2 Future research question

Could a combination of CATT and KIVI identify reservoir hosts?

A recent study has used the CATT to screen cattle and pigs (444) for the presence of *T. brucei s.l.* in the field prior to the collection of the samples. Using this method, animals with possible active infections of *T. brucei gambiense* can be identified and a blood sample taken for culturing using kits for *in* vitro isolation of trypanosomes (KIVI). Culturing the parasites would amplify their number and increase the DNA yield to allow for easier detection of single copy genes. Such an approach should overcome the shortfalls of the previous study and so provide a more reliable result. Ideally such a study design would be better trialled in an area where previous studies have found animal reservoirs of *T. b. gambiense*.

6.3.5 Final Conclusion

The results of the study show that although the detection of *T. brucei s.l.* in both the vector and local animal populations is relatively straightforward the ability to detect down to the sub-species level and identify *T. b. gambiense* is limited by the lack of highly sensitive diagnostic assays. This is demonstrated in the ~40% of *T. brucei s.l.* positive samples that lacked enough genetic material for single copy gene detection and in the inability to identify any *T. b. gambiense*. Therefore, there is a need to develop a highly sensitive assay that can detect *T. b. gambiense* from multiple sources including the vector and non-human vertebrate host animals.

The results of the LAMP assay are promising and have the potential of screening large numbers of tsetse for the presence of *T. brucei s.l.* however once more the assay required to identify down to the sub-species level lacks sufficient sensitivity. A sub-species specific test with an increased sensitivity would greatly benefit the current elimination goals.

Finally, the assessment of the impact of Tiny Targets on the transmission of salivarian trypanosomes was inconclusive due to confounding factors of sub-optimal numbers of cattle sampled and the inability to monitor where the cattle moved to in the intervening

period between rounds. However, based on the effects on tsetse catch numbers and the modelling data the Tiny Targets are a cost effective method of vector control that can complement the screening and treatment of the human population in endemic foci.

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Appendix

Illuminating the prevalence of *Trypanosoma brucei s.l.* in *Glossina* using LAMP as a tool for xenomonitoring.

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Abstract

Background

As the reality of eliminating human African trypanosomiasis (HAT) by 2020 draws closer, the need to detect and identify the remaining areas of transmission increases. Here, we have explored the feasibility of using commercially available LAMP kits, designed to detect the *Trypanozoon* group of trypanosomes, as a xenomonitoring tool to screen tsetse flies for trypanosomes to be used in future epidemiological surveys.

Methods and Findings

The DNA extraction method was simplified and worked with the LAMP kits to detect a single positive fly when pooled with 19 negative flies and the absolute lowest limit of detection that the kits were

able to work at was the equivalent of 0.1 trypanosome per ml. The DNA from *Trypanosoma brucei brucei* could be detected six days after the fly had taken a blood meal containing dead trypanosomes and when confronted with a range of non-target species, from both laboratory-reared flies and wild-caught flies, the kits showed no evidence of cross-reacting.

Conclusion

We have shown that it is possible to use a simplified DNA extraction method in conjunction with the pooling of tsetse flies to decrease the time it would take to screen large numbers of flies for the presence of *Trypanozoon* trypanosomes. The use of commercially-available LAMP kits provides a reliable and highly sensitive tool for xenomonitoring and identifying potential sleeping sickness transmission sites.

Author summary

Recent control efforts have reduced the global incidence of *Gambiense* human African trypanosomiasis (HAT) to <5,000 cases per year, strengthening the prospect of eliminating the disease as a public health problem by 2020. To meet this goal, new methods for identifying transmission must be explored to provide a cost-effective way of identifying hotspots and areas of re-emergence; commercial loop-mediated isothermal amplification (LAMP) kits that detect the trypanosome subgenus, responsible for the two forms of sleeping sickness, have been developed. The LAMP kits were tested to assess their sensitivity, specificity and suitability as a method of screening the vector of the disease, *Glossina*, for *Trypanozoon* infection, in xenomonitoring campaigns. A simplified DNA extraction process that worked in conjunction with the LAMP kits on pooled samples demonstrated a faster method of processing large numbers of flies compared to

other molecular tools. The kits performed well in our experiments and demonstrated the ability of detecting low levels of target DNA, equivalent to 0.1 trypanosome per ml. The lack of cross reaction with non-target species of trypanosomes makes the kits reliable in so far as they will only react with the *Trypanozoon* group of parasites of which the two human forms of the disease belong, however, further species-specific tests would need to be undertaken to identify HAT areas on selected samples.

Introduction

Human African trypanosomiasis (HAT), commonly known as sleeping sickness, is a parasitic disease caused by two sub-species of trypanosome, *Trypanosoma brucei gambiense* in West and Central Africa, which causes a chronic disease and *T.b. rhodesiense* in East and southern Africa which causes an acute disease. These pathogens are transmitted by several species of tsetse flies (*Glossina sp*.). Through active case detection and treatment, cases of *Gambiense* sleeping sickness are estimated to be ≤5000 as of 2014 (445). The greatest contribution to overall HAT burden is the west African (*Gambiense*) form of the disease as this accounts for 98% of all sleeping sickness cases in most recent estimates(56). This is reflected by the number of people at risk of the disease, with 57 million people at risk of *Gambiense* sleeping sickness compared to 12.3 million at risk of the *Rhodesiense* form (360). The methods of controlling HAT differ according to the underlying nature of the two forms, with more emphasis being placed on vector control in the case of *Rhodesiense* HAT. *Gambiense* HAT is generally considered to be anthroponotic and control is achieved mainly by screening and treating human cases; vector control has not played an important role. However with the development of more cost-effective methods (234) it seems likely that tsetse control will play an important role in efforts against *Gambiense* HAT. (446).

Detection of *Gambiense* HAT relies largely on active or passive screening of the population at risk. Active screening is difficult; screening >70% of a target population is seldom achieved (353) and

there are sensitivity issues as the card agglutination test (CATT) is not 100% accurate and is often one of a series of tests including lymph node puncture, fresh blood and thick blood film examination (355-357) . Moreover, as the prevalence of HAT decreases, the cost of detecting each case increases, leading to active screening programmes being scaled down or abandoned as other health priorities take precedent (361, 447).

An alternative to screening people is to screen vectors for the presence of the pathogen. This approach, termed xenomonitoring, has proven useful in the control of other vector transmitted diseases such as lymphatic filariasis (362). Rather than screening people, tsetse flies might be caught using simple traps and subsequently analysed for presence of *T. brucei s.l.*. By monitoring trypanosomes in tsetse, xenomonitoring would measure current rates of transmission in a defined area. By contrast, the chronic nature of *Gambiense* HAT and the mobility of humans mean that cases are detected months, if not years, after infection, and in places far removed from sites of transmission.

Hitherto, detection of pathogenic trypanosomes in individual tsetse flies relied largely on dissection and microscopic evaluation of individual flies which is technically demanding and time consuming (349). Molecular techniques are more sensitive than classical dissection, but they are also laborious, requiring skilled workers and well-equipped laboratories (364). Xenomonitoring for HAT requires novel methods that are easy, rapid, cheap, specific and sensitive. A method that offers the prospect of being fit for this purpose is coupling a rapid and robust method of extracting trypanosome DNA from a fly with a simplified molecular test such as loop-mediated isothermal amplification (LAMP). The LAMP test for HAT amplifies the repetitive insertion mobile element (RIME) of the *Trypanozoon* group (152) . LAMP is carried out at a constant temperature, thereby removing the need for thermocyclers, and results are read visually (150).

Studies of trypanosome infection in tsetse flies are generally concerned with quantifying the prevalence of pathogens in the vector population. For this purpose, the status of infection in individual flies must be quantified; only tsetse with a mature infection where *T. brucei s.l.* are

observed in the salivary glands are infectious. For xenomonitioring however, the aim is to detect the presence or absence of pathogens in a vector population. This specific aim offers two opportunities to improve the cost-effectiveness of screening. First, pooled groups rather than single individuals might be screened for the presence of pathogens. Second, a molecular method may be able to detect trypanosomes that have been recently ingested but which will not lead to a mature infection. Older tsetse flies are much less susceptible to infection with *T. brucei* (368). However, flies that are refractory to infection will nonetheless ingest trypanosomes from infected hosts and a sensitive method may be able to detect these transient trypanosomes in recently-fed flies.

Previous work has demonstrated the ability of an in-house LAMP assay to detect *T. brucei* in laboratory-infected tsetse and in pooled groups of tsetse (154). Here, we assessed various simple methods for (i) extracting trypanosome DNA from tsetse, and (ii) the potential of using a commercially-available Loopamp™ *Trypanosoma brucei* detection kit developed by Eiken Chemical Co (Japan). Performance of LAMP was assessed with laboratory flies experimentally infected with trypanosomes and wild-caught tsetse flies from an area of northern Uganda where *T. brucei s.l.* is present.

Methods

Tsetse fly samples were tested using the Loopamp™ kit to determine the presence of trypanosome DNA, and the limit of detection of LAMP. DNA extraction methods were optimised for field settings and tested with the LAMP kits in pooled fly assays. The specificity of the kit was tested against flies infected with non-target species of trypanosomes, most likely *Trypanosoma congolense sl.* and *Trypanosoma vivax* in both laboratory-infected (*Glossina morsitans morsitans*) and wild flies (*Glossina fuscipes fuscipes*).

DNA Extraction

Tsetse flies were dissected and their midguts stored in 60µl of 100% ethanol (EtOH). At the Liverpool School of Tropical Medicine (LSTM) a standard DNA extraction procedure, hereafter referred to as the Chelex method, was followed: 70ul of distilled water was added to the midgut sample followed by centrifugation at 13,000rpm (15sec) and removal of 100µl of supernatant. Tissue samples were washed three times by adding and removing 100µl of distilled water. To the washed tissue a 100µl suspension of Chelex and Proteinase K (20mg/ml) was added to give a final concentration of 1% Chelex, and incubated at 56˚C for 1 h. The sample was then incubated for 30 minutes at 93˚C, centrifuged at 13,000rpm (15sec), the supernatant removed, and stored at -20C.

Simplification of DNA extraction

The current Chelex DNA extraction method, although robust, is not field-friendly and takes ~2 h to complete with a number of steps and components, some of which require a cold chain. To use this kit in the field, the current method needs to be optimised to ensure it is rapid, robust and simple. Five alternative DNA extraction methods aimed at reducing the time and complexity of the current Chelex extraction method for use in field studies were designed (Table 1).

Table 1. Details of five alternative field-friendly DNA extraction methods.

Method	Spin 13,000 rpm	Wash x3	Spin 13,000 rpm	5 % Chelex or 1% TE	Proteinase K	Incubation		Total Time (Min)
						56˚C	93˚C	
Chelex	Y	Y	Y	Chelex	Y	60 m	30 m	120
1/2 time chelex	Y	Y	Y	Chelex	Y	30 m	15 m	50
1/4 time chelex	Y	Y	Y	Chelex	Y	15 m	7.5 m	30
TE wash off alcohol	Y	Y	Y	TE			15 m	45
TE with chelex	Y	*		Chelex			15 m	15
TE leave in alcohol	Y			TE			15 m	22

*The alcohol was not washed off, instead the samples were transferred out of the EtOH and into a clean empty tube

These different DNA extraction methods were tested against two sets of trypanosome concentrations at 10^2 and 10^4 trypanosomes per ml, with each of these concentrations being tested in eight replicates with a combined total of 16 replicates across the two concentrations.

Amplification tests

LAMP

The RIME LAMP test was performed using the Loopamp™ *Trypanosoma brucei* Detection Kits manufactured by Eiken Chemical Co.,Ltd, Japan, according to the manufacturer's specifications. Briefly, 2.5µl of template DNA was added to 22.5 µl of nuclease-free water. LAMP reagents were reconstituted in the lids of the tubes, and after two minutes incubation, the tubes were inverted five times. A thermocycler was used to heat the samples for forty minutes at 65˚C and then at 85˚C for five minutes to stop the reaction. Results were determined by visualising presence or absence of fluorescence in the reaction tubes on a UV gel imager, and each sample was classed as positive or negative based on two separate blind screenings by two of the co-authors (LJC, JKL).

PCR

Alongside the RIME LAMP assays, samples were also amplified using the TBR *Trypanozoon* primers and a nested universal trypanosome ITS primer set(147) (Table 2).

Table 2. Primers used in this study.

Primer name	Target species	Primer sequence 5'-3'	Published/designed by
TBR Forward	*T. brucei s.l.*	TGCGCAGTTAACGCTATTATACA	Matovu
TBR Reverse	*T. brucei s.l.*	AAAGAACAGCGTTGCAAACTT	Matovu
Tryp 1	*Trypanosomatidae*	AAGCCAAGTCATCCATCG	Adams et al. 2006

Tryp 2	*Trypanosomatidae*	TAGAGGAAGCAAAAG	Adams et al. 2006
Tryp 3	*Trypanosomatidae*	TGCAATTATTGGTCGCGC	Adams et al. 2006
Tryp 4	*Trypanosomatidae*	CTTTGCTGCGTTCTT	Adams et al. 2006

The PCR reactions consisted of 12.5 µl MyTaq Red Mix (Bioline), 1 µl of forward and reverse primer (25mmol), 8.5 µl of nuclease free water and 2 µl of DNA template. For the TBR primers the PCR cycles were: an initial denaturation step at 93˚C for 2 mins, followed by 35 cycles at 94˚C for 10 s, 55˚C for 10 s and 72˚C for 10 s with a final extension step of 30 s at 72˚C, for the nested PCR 1 µl of PCR product from the first nest was added to the second to act as the template.

For both nests of the ITS primers the PCR cycle had an initial denaturation at 95˚C for 5 mins followed by 35 cycles of 94˚C for 15 s, 54˚C for 15 s and 72˚C for 10 s with a final extension step at 72˚C for 5 mins.

Limit of Detection

Colony-reared *G. m. morsitans* were dissected three days after their first blood meal, the midguts were collected into individual tubes and preserved in 100% EtOH. A trypanosome dilution series was created by first making a stock concentration of $2x10^5$ trypanosomes per ml using a haemocytometer (20). The trypanosomes were then heated at 93˚C for 30 mins to lyse the parasites and extract the DNA. Using the eluted DNA, a tenfold dilution series was then created with the equivalent number of parasites for each gradient step as follows:

$2x10^5$/ml (stock), $2x10^4$/ml, $2x10^3$/ml, $2x10^2$/ml, $2x10^1$/ml, 2/ml, 0.2/ml and 0.02/ml.

To prepare the samples for analysis a modified version of the Chelex method was used. To remove residual EtOH from the preserved tsetse tissue, midguts were washed three times as previously described. To each washed midgut, 50µl of a trypanosome DNA concentration was added. Following the addition of trypanosome DNA, 50µl of a Chelex suspension (10% Chelex with a 2% proteinase K concentration) was added to the midgut tissue for a final volume of 100µl. The DNA concentration

series represents $1x10^5$/ml, $1x10^4$/ml, $1x10^3$/ml, $1x10^2$/ml, $1x10^1$/ml, 1/ml, 0.1/ml, 0.01/ml trypanosomes suspended in 5% Chelex suspension/1% proteinase K. Each DNA dilution was processed according to the standard Chelex extraction method. Table 3 summarises the number of trypanosomes in the DNA dilution series per ml, per 100µl, and per reaction for both the LAMP (2.5µl of template) and PCR (2µl of template) assays.

Table 3. A breakdown explaining the equivalent number of trypanosomes at different volumes used in the study.

	Equivalent number of trypanosomes present per DNA concentration at:							
	1x10^5/ml	**1x10^4/ml**	**1x10^3/ml**	**1x10^2/ml**	**1x10^1/ml**	**1/ml**	**0.1/ml**	**0.01/ml**
Trypanosomes ml	100000	10000	1000	100	10	1	0.1	0.01
per 100µl sample	10000	1000	100	10	1	0.1	0.01	0.001
per LAMP assay	250	25	2.5	0.25	0.025	0.0025	0.00025	0.000025
ng/µl LAMP assay	$2.5x10^{-2}$	$2.5x10^{-3}$	$2.5x10^{-4}$	$2.5x10^{-5}$	$2.5x10^{-6}$	$2.5x10^{-7}$	$2.5x10^{-8}$	$2.5x10^{-9}$
per PCR assay	200	20	2	0.2	0.02	0.002	0.0002	0.00002
ng/µl TBR assay	$2x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$	$2x10^{-5}$	$2x10^{-6}$	$2x10^{-7}$	$2x10^{-8}$	$2x10^{-9}$

Pooling

Infected *G.m. morsitans* flies were generated by adding 200µl of thawed *T.b. brucei* GFP J10 blood stabilates to five ml of defibrinated horse blood (TCS Biosciences Ltd). Teneral flies were fed through a silicon membrane placed over the blood which was heated to 37˚C. After seven days the flies were dissected and their midguts visually screened for infection using a compound microscope. Positive midguts were then stored individually in 100% EtOH and later they were processed using the chosen simplified DNA extraction method. Parallel to the infected flies a group of uninfected flies were also generated apart from the omission of the 200µl of *T. b. brucei* blood stabilate to the five ml of horse blood. The midguts of negative flies were screened prior to being stored in the EtOH with microscopy and also later, after DNA extraction using the Chelex method, with TBR PCR to ensure they were truly uninfected.

Pools of tsetse flies were generated by adding 3µl of DNA template from a single positive fly sample to pools of four, nine and nineteen uninfected fly samples, in which each fly contributed 3µl of DNA extract to the pool. A total of 9 replicates were performed per pool and each pool was tested with LAMP.

Persistence of DNA in tsetse material

To assess the ability of the Loopamp^{TM} kit to detect trypanosomes from a previous blood meal 200µl of a 10^6/ml *T. b. brucei* blood stabilate was incubated for 15 minutes at 54˚C to kill the trypanosomes and hence prevent establishment of an infection in the tsetse. The heat-killed trypanosomes were added to 5ml of defibrinated horse blood. The viability of the parasites was determined through microscopy and the use of the stain Trypan blue(372). If no living trypanosomes were observed the blood was then fed to teneral flies. A similar volume of uninfected blood was heated and fed to a control group of flies. Every 24 hours after the initial blood meal, three experimental flies and one control fly were dissected and the midgut removed and stored in 95% EtOH. During each dissection

the midgut samples were screened visually for living trypanosomes using a compound microscope as a secondary measure to ensure no trypanosomes had survived and had started to infect the flies. The midgut samples were processed using the Chelex method followed by LAMP and PCR assays as described. The experiments were repeated until the LAMP and PCR tests showed two consecutive days of negative results in all experimental fly samples.

Mixed infections

LAMP kits were tested for cross reactivity with single and mixed species infections of both *T. congolense* (1/148) and *T.b. brucei* (GFP J10). Flies were infected by artificial membrane feeding (373). Partially fed flies were removed. On the seventh day post-infection all flies were dissected and their midguts screened for trypanosomes by microscopy (448).
The four groups were processed using the Chelex method and analysed using the *Trypanozoon* specific TBR primers and the universal ITS primers alongside the RIME LAMP kits.

Wild-caught tsetse flies

From NW Uganda, 449 *G.f. fuscipes* were caught and screened by microscopy, universal ITS primers and the LAMP kits. The flies were caught from April to June 2013 from eight trap sites (Northings 381161-383674, Eastings 276506-287545) in the district of Koboko. The flies were dissected in the field and their mouthparts, salivary glands and midguts were separately screened for trypanosomes by microscopy. All samples were then stored individually in 100% EtOH and shipped at room temperature to LSTM where they underwent the Chelex extraction prior to PCR and LAMP analysis.

Results

Simplification of DNA extraction

The results for the six different DNA extraction methods, tested with the LAMP kits, are shown in Table 4. The method that produced the highest number of positive results was ‘½ time Chelex

'followed by 'TE with Chelex' detecting 13 and 12 positive samples respectively out of a total of 16 positives. The lowest scoring methods were 'TE Leave in alcohol' and 'TE wash off alcohol'. Taking into account the number of steps involved for each method and the total time it takes 'TE with Chelex' was deemed the most efficient as it took half the time of '½ Chelex' but only identified one less positive sample (Table 4).

Table 4. Overview of the results for the different DNA extraction methods.

DNA Extraction methods	Positive results (trypanosome/ml)		Total number of positives	% of positive samples identified
	10^2	10^4		
Chelex	4	7	11	69
½ time Chelex	6	7	13	81
¼ time Chelex	5	6	11	69
TE Leave in alcohol	2	5	7	44
TE wash off alcohol	1	8	9	56
TE with Chelex	8	4	12	75

Limit of Detection

The results (Figure 1) shows that the LAMP kit was able to detect 100% of the spiked samples up until 10^4 trypanosomes per ml, after which there was a decline in the ability to detect the trypanosome DNA until 10^{-1} trypanosomes per ml. The sensitivity of the LAMP kit when compared with the TBR PCR was better by a factor of two, as the TBR PCR had a limit of detection of 10^1 trypanosomes/ml. Although the template volumes for the LAMP and TBR assays varied slightly by

0.5 µl (or 25%) this variation is too small to explain the two fold, (or 100 x) greater difference in the sensitivity of the two assays.

Fig 1. Limit of detection results for LAMP and TBR primers, a total of six flies were used for each dilution gradient for both assays.

Pooling

Pooling experiments show that LAMP was able to amplify one positive midgut in pools of four, nine and 19 uninfected midguts - this was repeated in nine independent tests. There were two kit failures, one in ratio 1:5 and the other in 1:20. The failure was caused by the dried reagents failing to properly dissolve and integrate into the reaction.

DNA Persistence

The number of tsetse that were able to give a positive LAMP results declined from 100% after 48 hours to just 11% by day 6 (Fig 2).

Fig 2. Persistence of *T. b. brucei* DNA in *G. m. morsitans*

Mixed infections

Thirteen out of sixteen surviving flies from a mono *T.b. brucei* infection were identified as positive by both microscopy and TBR PCR (Table 5). When the same samples were tested with LAMP, all 16 flies tested positive. Of the 17 surviving flies from the *T. congolense* single infection, 14 were positive by microscopy, 13 tested positive with the ITS primers (which amplifies and differentiates both *T. brucei and T. congolense)* and none were detected by LAMP, which is specific for *T. brucei s.l.*. Of the mixed infection group, 15 flies were positive by microscopy, 18 tested positive using the generic ITS primers, 17 tested positive with the *brucei*-specific TBR primers and 20 tested positive with the LAMP kits. Of the 18 flies identified as positive with the ITS PCR, one had a single *T. congolense* infection, 3 had single *T. b. brucei* infections and 14 had mixed infections. Both the TBR PCR and LAMP did not cross-react with the single *T. congolense* infection (Table 5). The negative control group tested negative with microscopy, PCR and LAMP assays. LAMP is shown to be more sensitive than either the ITS or TBR primer sets.

Table 5. The results of testing cross reactivity with flies containing single and mixed infections of *T.b. brucei* and *T. congolense.*

Infection	Assay	Positive	Negative	Lost Flies	Total
Single *T. brucei* infection	LAMP	16	0	9	25
	TBR	13	3	9	25
	Microscopy	13	3	9	25
Single *T. congolense* infection	LAMP	0	17	8	25
	ITS	13	4	8	25
	Microscopy	14	3	8	25
Mixed infection *T. b. brucei* and *T. congolense*	LAMP	20	3	2	25
	TBR	17	6	2	25
	ITS	18	5	2	25
	Microscopy	15	8	2	25
Negative control	LAMP	0	16	9	25
	TBR	0	16	9	25
	ITS	0	16	9	25
	Microscopy	0	16	9	25

Wild flies

Of the 449 wild-caught tsetse, microscopy identified one salivary gland positive *T. brucei s.l.* sample, the universal ITS primers identified two *T. brucei* sl. samples while LAMP identified six. The ITS assay identified 40 flies positive for non-*Trypanozoon* trypanosomes (*T. vivax* n:11, *T. congolense s.l.* n:5, *T. grayi* n: 22, *T. simiae* n:2) which the LAMP kits did not cross-react with.

Discussion

This study has demonstrated the potential of the Loopamp™ *Trypanosoma brucei* detection kit as a tool for identifying *Trypanozoon* DNA in tsetse. The LAMP kits showed a high degree of specificity, with no cross-reaction when challenged with non-target species of trypanosomes. LAMP was more sensitive than standard PCR in both laboratory-infected and wild-caught tsetse flies. Further we show that a single trypanosome-infected tsetse could be detected in a pool of 20 flies. Simplified DNA extractions were developed and the results show the ability to reduce the time of DNA extraction, although moderate equipment and skill is still required. The results suggest that LAMP may be a useful tool for epidemiological surveillance of *T. brucei s.l.* in HAT endemic regions to estimate the burden of the disease (in combination with species-specific PCR). This may be more cost-effective than active detection of human cases, and would allow control programmes to prioritise areas for HAT control.

The field extraction methods tested here have been assessed by balancing the length of the process, reliability at detecting positive samples and the number of steps involved in the method, which is a proxy for its complexity. The most successful method at identifying spiked samples was the '½ time Chelex method' but once the complexity and time was taken into account 'TE with Chelex' was decided as the most efficient DNA extraction method. However, all of these extractions still require access to centrifuges, multiple reagents and handling steps and must be improved before implementation.

In previous studies, LAMP assays on pooled samples showed a decline in sensitivity by 60% with a ratio of 1:15 (154). In the present study, the LAMP kit was capable of detecting one infected tsetse amongst 19 uninfected flies 100% of the time when coupled with the TE Chelex extraction method. This demonstrates that both the kit and the TE Chelex extraction have good potential for use as tools in the xenomonitoring of tsetse.

The serial dilution experiment confirms that the LAMP kits have an extremely high sensitivity in detecting trypanosomes. Previous studies have reported limits of detection ranging from the

equivalent of 100 to 0.001 trypanosomes per ml (152, 154, 364) using in-house LAMP assays in accordance with results shown here. The advantage of using a commercially available, standardised LAMP kit would allow researchers to compare different settings with each other without differences in method confounding the results. The ability of the LAMP kits to detect such a low number of trypanosomes per ml is advantageous for its use in xenomonitoring as flies actively transmitting the disease can have infections ranging from $<1 \times 10^1$ to 1×10^4 depending on the stage of infection(374).

Another, less obvious, advantage with xenomonitoring is that the tsetse flies are continuously feeding on a wide range of local vertebrates(290) and could, in effect, act as an efficient source of blood for screening in order to detect *Trypanozoon* species in the environment. If tsetse are able to pick up *T. brucei s.l.* trypanosomes in blood meals and trypanosome DNA can be amplified, it would be possible to detect if HAT is present in a region. The results for *T. b. brucei* DNA persistence are highly encouraging as they demonstrate that it is possible to detect DNA from a blood meal 100% of the time after 48 hours post-feeding and in the extreme we have shown it is possible for the DNA to remain in a detectable state for up to six days in our colony flies. This is despite the fact that the tsetse flies in our experiment were not only digesting the initial infected blood meal, but they subsequently took on fresh blood, at later feeds, which helped flush their digestive system of previous meals.

The situation in the field may also be complicated by the presence of other trypanosome co-infections in the tsetse flies (254). The results from the co-infection experiments demonstrate that even with co-infected flies it was still possible to identify those that had a *T.b. brucei* infection and in the one case where the fly cleared the *T.b. brucei* infection but maintained a *T. congolense* infection, the LAMP kit recorded this as a *T.b. brucei* negative fly.

The specificity of the LAMP kits was good when tested against single infections of *T. congolense* and *T.b. brucei*, with no cross-reaction with the flies infected with *T. congolense*.

In wild-caught flies, the LAMP test was more sensitive than the PCR based method, and was able to identify more *T. brucei s.l.* infections in the 449 flies caught from Uganda.

The LAMP kit used in the present study is only able to identify the *Trypanozoon* group, meaning that a species-specific PCR would still be necessary to differentiate human infective forms from the other trypanosomes in order to guide HAT control programmes effectively. Unfortunately the species-specific PCRs used to differentiate the *Trypanozoon* group use single-copy gene targets, making them significantly less sensitive than LAMP and other PCRs (e.g., TBR, ITS). In addition, it is currently not possible to conduct LAMP in a high-throughput manner, and the cost of kits is still significant, at $2.60 per test. A larger study involving cost analysis of PCR vs LAMP should be conducted to verify that molecular xenomonitoring is a suitable tool for studying the epidemiology of HAT and is able to highlight hotspot areas of transmission.

Conclusion

This study has demonstrated a robust detection limit of the Loopamp™ *Trypanozoon* detection kit of 0.1 trypanosome per ml in dissected tsetse midguts. We demonstrated that the kit has high specificity, with no cross-reactivity in flies with multiple infections, including wild caught flies that had a greater variation of non-target species and provided a more realistic challenge. The kits could also detect *T.b. brucei* DNA 6 days after consumption of a contaminated blood meal, despite having fresh feeds every 48 hrs, which would be flushing their digestive system with clean blood. The sensitivity of the kits was high enough to allow for the detection of a single infected fly in a pool of 20 flies. The next step would be to test the DNA extraction and pooling methods in the field alongside a wider cost and feasibility evaluation of LAMP vs PCR and the ability to use xenomonitoring of HAT over time in the elimination campaign.

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